The Roles of Dicer and TRBP in HCV Replication

A Thesis Submitted to the

College of Graduate Studies and Research

in Partial Fulfillment of the Requirements for the

Master of Science Degree in the

Department of Microbiology and Immunology

University of Saskatchewan

Saskatoon, SK

S7N 5E5 CANADA

By

Chao Zhang

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master of Science degree from the University of Saskatchewan, the author agrees that the libraries of this University may make it freely available for inspection. The author further agrees that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised the thesis work or, in their absence, by the Head of the Department or the Dean of the College in which the thesis work was done. It is understood that any copying or publication or use of this thesis or parts of it for financial gain shall not be allowed without the author's written permission. It is also understood that due recognition shall be given to the author and to the University of Saskatchewan in any scholarly use which may be made of any material in this thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Microbiology and Immunology Department

Health Science Building, 107 Wiggins Road

University of Saskatchewan

Saskatoon SK

S7N 5E5 CANADA

ABSTRACT

MicroRNAs (miRNAs) are non-coding small RNAs that regulate eukaryotic gene activity at the post-transcriptional level by a process termed miRNA gene suppression. MicroRNA-122 (miR-122) is predominantly expressed in human liver cells and recent studies indicated that miR-122 promotes Hepatitis C Virus (HCV) replication and translation through physical interaction with two tandem binding sites located in the 5' untranslated region (5'UTR) of the HCV genome (Jopling, *et al*., 2006; Jopling, *et al*., 2008). It has been reported that host genes that are also implicated in the miRNA gene suppression pathway are key regulators of HCV replication (Randall, *et al*., 2007). Two proteins, Dicer, a key RNaseIII enzyme, and its binding partner TRBP are essential proteins for miRNA activity. They are part of a protein complex called the RNA induced silencing complex (RISC) which also includes Argonaute proteins, and function in miRNA biogenesis loading the miRNA into RISC. As such, they are intriguing targets to study host-viral interplay during HCV replication.

In our study, we designed siRNAs to knock down Dicer and TRBP and then observed the effects of gene knockdown on full length J6/JFH-1-RLuc HCV (genotype 2a chimeric genome) replication and translation. The results showed that knocking down Dicer and TRBP reduced wild type (wt) J6/JFH-1-RLuc replication but had almost no effects on HCV translation in human liver cells. However, since knocking down Dicer and TRBP did not significantly alter miR-122 levels in the cell, it appears that the role of Dicer and TRBP was not solely the biogenesis of miR-122. This was confirmed by an experiment in which we observed that knocking down Dicer and TRBP also attenuated replication of

a mutant virus in which replication is dependent on a exogenously supplied miRNA instead of endogenous miR-122.

Taken together, the results supported the hypotheses that Dicer and TRBP facilitate HCV infection mainly through HCV replication but not translation. The effects of Dicer and TRBP on HCV replication are not solely due to miR-122 biogenesis, and may be due to RISC loading functions in steps of miRNA gene suppression.

This study has set some essential groundwork for investigating potential roles of host factors in the RNAi machinery modulating HCV replication/translation and exploring novel antiviral targets.

ACKNOWLEDGEMENTS

I would first like to express my sincere gratitude to my supervisor Dr. Joyce Wilson for providing me with a great research opportunity. I appreciate her great guidance not just because I have gained a wealth of professional knowledge and a variety of techniques performed in the laboratory, I appreciate Joyce's training ways to become an independent and creative researcher. I was always encouraged to be exposed to cutting-edge information, learn new techniques and solve problems on my own initiative. I would also like to acknowledge my advisory committee members, Dr. Wei Xiao, Dr. Sylvia Van Den Hurk, and Dr. Peter Howard as well as my external examiner Dr. Troy Harkness. Thanks for their critical comments and constructive suggestions during the whole research period.

I could not have made it through the past three-year oversea study without the help and company of Adam Huys, Patricia Thibault and all the people in our big laboratory, even the researchers at VIDO. They were always there for me, willing to answer every single question I had and to patiently go through any technical problem with me. Special thanks to the staff at VIDO for creating a warm friendly environment to work in and thanks to VIDO to providing the researchers with such convenient, excellent resources and facilities.

Thanks must also go out to financial support for my project funded by the National Sciences and Engineering Research Council (NSERC) of Canada and College of Medicine.

Last, but not least I would like to acknowledge my dear parents Yanming Li and Feng Zhang. It was only because of their selfless love, continuous support and encourage that I was able to become what I am today.

All these paved the way for what I know, what I gain and what I am today. Thank you and dedicate this thesis to all the people I deeply appreciate.

TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
Ago	Argonaute
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
ddH_2O	Double distilled water
DEPC H_2O	Diethyl Pyrocarbonate water
DMSO	Dimethyl Sulfoxide
dsRNA	Double stranded RNA
DMEM	Dulbecco's Modified Eagle's Medium
D-PBS	Dulbecco s Phosphate Buffered Saline
dsRBD	Double-stranded RNA binding domain
EDTA	Ethylenediamine tetra-acetic acid
E. coli	*Escherichia coli*
FBS	Fetal bovine serum
FLuc	Firefly Luciferase
FXMR	Fragile X mental retardation
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HCV	Hepatitis C Virus
Huh7 cell lines	Human hepatoma 7 cell lines
INF	Interferon
IRES	Internal ribosome entry site
JFH-1	*Japanese fulminant hepatitis 1*
Kb	Kilobase pair
KDa	Kilodalton
mut	Mutant
min	Minute (s)

miRNA	MicroRNA
mRNA	Messenger RNA
miRNPs	MiRNA ribonucleoproteins
NS	Non-structural
ORF	Open reading frame
PACT	Protein activator of PKR
PKR	Protein Kinase R
PBS	Phosphate-Buffered Saline
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PAZ	Piwi Argonaute Zwille
p.s.e.	Post second electroporation
rcf	Relative Centrifugal Force
RISC	RNA Induced Silencing Complex
RNPs	Ribonucleoproteins
RNAi	RNA Interference
rpm	Rotation per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RLuc	Renilla Luciferase
siRNA	Small interferente RNA
SSC	Sodium chloride-Sodium *citrate*
Sec	Second (s)
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TRBP	Human immunodeficiency virus 1 (HIV-1) transactivating response (TAR) RNA-binding protein
UTRs	Untranslated regions
U6 snRNA	Small nuclear RNA U6
V	Volt
wt	Wild type

1.0 INTRODUCTION

1.1 Hepatitis C

The disease caused by Hepatitis C virus (HCV) was first observed in the 1970s and was characterized as post-transfusion hepatitis that was not caused by Hepatitis A or B virus (thus termed non-A and non-B hepatitis). Hepatitis C virus was identified as the cause of this disease by using recombinant DNA technology in 1989 (Choo, *et al.* 1989), since then, HCV has been recognized as a major cause of chronic liver disease and affects nearly 200 million people worldwide. In Canada, about 250,000 people are currently infected, and nearly 8000 individuals become newly infected each year (http://www.hc-sc.gc.ca/hl-vs/iyh-vsv/diseases-maladies/hepc-eng.php). Persistent HCV infection is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma. In general, HCV infection results in an acute infection which is asymptomatic and often undiagnosed. Approximately 15-20% of acutely infected patients are able to self-clear the infection without mediation. However, in the majority of cases, acute infection becomes chronic. Approximately one-fifth of chronically infected individuals progress to cirrhosis over a 5-30 year period and a small number of individuals develop hepatocellular carcinoma (Chen, *et al.,* 2006). HCV is generally transmitted through blood transfusions, hemodialysis, organ transplants, and sharing of needles (Lauer, *et al.,* 2001).

1.2 HCV Therapeutics

So far the development of anti-HCV vaccines has been unsuccessful mainly due to the genetic variability of HCV which allows the virus to rapidly escape immune surveillance. Current clinical treatments against HCV infections are limited to a combined administration of pegylated interferon (INF)-α and ribavirin. However, this standard treatment regimen assures a long term eradication of HCV in less than half of infected patients and results in considerable side effects that include depression, hemolytic anemia, fever and chills, and produces different sustained viral response rates among different genotypes (Qureshi, *et al*., 2007). Moreover, the clinical development of novel antiviral drugs targeting HCV protein processing and replication is progressing more slowly than expected due to safety issues and viral resistance (Pawlotsky, *et al*., 2007). Thus, a detailed understanding of virus-host interaction during HCV infection may provide opportunities to develop alternative and more effective approaches to overcome viral resistance which will complement current antiviral therapies.

Our studies on virus-host interactions focus on the relationship between HCV infection and the host miRNA pathway. Several lines of evidence indicate that the miRNA pathway influences HCV replication. Results of a systematic RNA interference (RNAi) screen demonstrated that various cellular proteins, including essential components of the RNA interference pathway, were able to alter HCV infection (Randall, *et al*., 2007). At the same time, our lab had also discovered that depletion of Argonaute 2, a central effector of miRNA activity, affected HCV replication (unpublished). In addition, a liver specific micro RNA, miR-122, was identified to bind to a region within the 5'UTR (Figure 1-1) and facilitate HCV RNA accumulation and translation through

unknown mechanisms (Jopling, *et al*., 2006; Jopling, *et al*., 2008; Henke, *et al.,* 2008). Moreover, there is growing evidence that the reason why the host liver environment is highly permissive for HCV replication might be due to high levels of microRNA-122 (miR-122), and the host may specifically alter miR-122 levels as a mechanism to resist HCV replication as levels of miR-122 were downregulated following INF-β treatment (Pedersen, *et al*., 2007). Therefore, we were inspired to further explore the mechanisms of miRNA pathway enhancement of HCV replication, particularly focusing on proteins involved in the processing and activity of miRNAs, Dicer and TRBP, with the goal of identifying novel antiviral targets.

1.3 Hepatitis C Virus

HCV belongs to *Hepacivirus* genus within the *Flaviviridae* family. The virus exists as six major genotypes, each being defined by their nucleotide sequences, which may differ by up to 30-35%. Each genotype is also divided into several subtypes (represented by letters) based on sequence differences (Moradpour, *et al.,* 2007). HCV genotypes have varied geographical distributions with genotype 1, 2, and 3 being widely distributed throughout Western countries and the Far East (Japan, China, Taiwan, and Thailand). Type 5 and 6 are the most prevalent in South Africa and Southeast Asia, respectively. Type 4 is predominant in the Middle East and Central Africa (McOmish, *et al.,* 1994). HCV is a spherical, enveloped virus 40-70 nm in diameter, with a narrow host range consisting of human and chimpanzees. HCV contains a 9.6 kb linear positive single-stranded RNA genome that encodes a single open reading frame (ORF), flanked by 5′ and 3′ untranslated regions (UTRs) (Farci, *et al.,* 2002). The 5'and 3'UTRs are the most conserved regions of HCV RNA and play key roles in regulating translation and

RNA replication. The 5' UTR contains a highly conserved internal ribosome entry sequence (IRES) onto which the 40S ribosomal subunit binds to initiate polyprotein translation in a cap-independent manner (Spahn, *et al.,* 2001). The 3' UTR is composed of a short variable region, a poly (U/UC) tract with average length of 80 nt, and a highly conserved 98 nt X-tail region all of which are essential for viral replication (Tanaka, *et al.,* 1996).

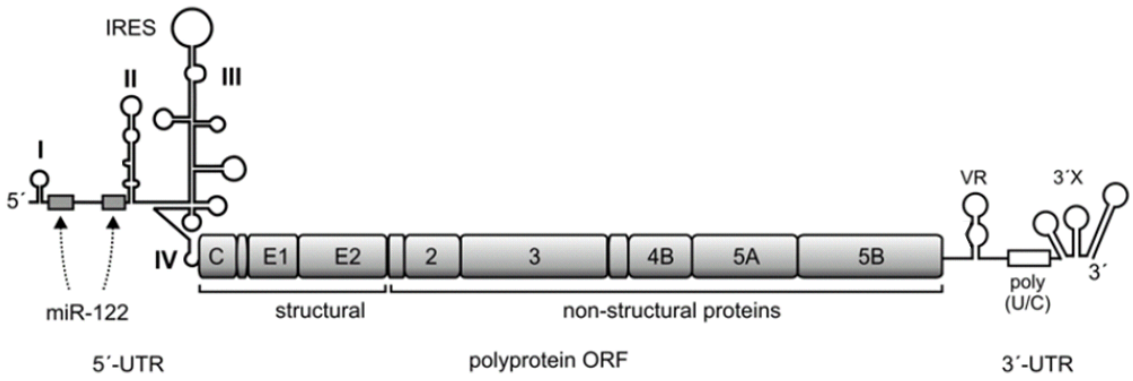


Figure 1-1: Genetic organization of HCV RNA genome (Jopling, *et al*., 2006).

The HCV genome is shown with the polyprotein ORF coding for the structural and the non-structural replication protein, the 5' and 3'UTRs. Sequences in the 5'UTR complementary to miR-122 are shown as grey boxes.

1.4 HCV Lifecycle:

1.4.1Attachment and Entry

HCV infection is a highly dynamic process with a virion half-life of only a few hours. HCV virions circulate in three forms, in physical association with very-low-

density lipoprotein (VLDL), or low-density lipoprotein (LDL); as free virions, or virions bound to immunoglobulins (Andre *et al*. 2005). Entry into hepatocytes requires multiple cell surface protein receptors: the tetraspanin CD81 (Pileri, *et al*. 1998), the scavenger receptor class B type I (SR-BI) (Scarselli, *et al*. 2002), and the tight junction proteins claudin-1 (Evans, *et al*. 2007) and occludin (Ploss, *et al*. 2009). HCV entry is also facilitated by additional receptor components, such as glycosaminoglycans, the LDL receptor, and the lectins DC-SIGN and L-SIGN, however, these are not essential entry factors and do not confer tissue specificity (Lozach, *et al*. 2003; Saunier, *et al*. 2003; Agnello, *et al*. 1999). Once attached, HCV is internalized via clathrin-mediated endocytosis and the viral nucleic acids are released into the host cell cytoplasm, following acidification of early endosomes (Evans, *et al*. 2007).

1.4.2 IRES Mediated Translation Initiation

HCV translation is the first macromolecular synthetic event after the HCV genome is released into the cytosol. In contrast to eukaryotic protein synthesis in which translation initiation involves cap-dependent scanning to arrest the start codon with canonical initiation factors, the naturally uncapped viral RNA genomes of HCV are translated via a cap-independent IRES-mediated mechanism. Initiation of IRES translation follows direct recruitment of ribosomes to the site of translation initiation with only a minimal requirement for canonical translation apparatus (Tsukiyama-Kohara, *et al*. 1992). The HCV 5'UTR comprises 341 nucleotides upstream of the translation initiation codon and contains four highly structured RNA domains (I-IV). Domains II, III, and IV, together with the first 24-40 nts of the core-encoding region, constitute the IRES

(Moradpour, *et al*., 2007). IRES-mediated translation is initiated by direct binding of the 40S ribosomal subunit to the HCV IRES. The IRES-40S ribosome complex sequentially recruits eukaryotic initiation factor eIF3 to stabilize ternary complex Met-tRNA-eIF2-GTP, resulting in the formation of a 48S rRNA complex at the AUG initiation codon. Finally, the 60S subunit is recruited to form an active 80S complex in a GTP hydrolysis step (Fraser, *et al*., 2007; Hellen, *et al*., 2009).

Translation of the HCV ORF yields a single polyprotein of approximately 3,000 amino acids. The polyprotein is co- and posttranslationally processed by cellular and viral proteases into at least 10 structural and nonstructural proteins (NS) (De Francesco, *et al*., 2000). The structural proteins, which form the viral particles, include core, and the two envelope glycoproteins E1 and E2. The non-structural proteins include p7, an ion channel; NS2-3, a protease; NS3, a serine protease and RNA helicase; NS4A; NS4B; NS5A, and NS5B, an RNA-dependent RNA polymerase (RdRp) (Fraser, *et al*., 2007) (Figure 1-1).

1.4.3 RNA Replication

HCV translation and replication occurs in association with intracellular membranes. Infection with HCV leads to visible alterations to host cellular ER membranes to form what is termed a "membranous web" (Egger, *et al*., 2002). The replication complex, composed of viral proteins, cellular components, and replicating RNA (Egger, *et al*., 2002) was identified as being specifically located within the membranous web (Gosert, *et al*., 2003). So far, the precise mechanisms of HCV replication are still poorly defined. As with other positive-strand RNA viruses, HCV

replication is assumed to be performed in two semiconservative and asymmetric steps, catalyzed by NS5B RdRp. It starts with the synthesis of a complementary negative-strand intermediate by using the viral genomic RNA as a template. In the second step, (-) strand RNA is used as a template to produce numerous strands of positive polarity, which is transcribed at a level in 5- to 10- fold excess compared with (-) RNA. Nascent (+) viral RNAs are subsequently subjected to polyprotein translation, synthesis of new intermediates of replication, or packaging into new virus particles (Suzuki, *et al*., 2007). In recent studies, Jopling et al. found that highly abundant and liver-specific microRNA-122 binds to a region within the 5'UTR and facilitates HCV replication through unknown mechanisms (Jopling, *et al*., 2006; Jopling, *et al*., 2008).

1.4.4 Virus Assembly and Release

Much has been learned about HCV assembly and release since the development of an efficient cell culture model system for HCV in 2005. Based on current evidence, assembly initiates on the cytosolic side of the ER membrane and maturation is completed in the ER lumen prior to release from host cells. In the early step of assembly, progeny genomes are released from the membranous web and moved to specialized lipid droplets (LDs). LDs are coated with viral proteins (Miyanari, *et al*., 2007; Boulant, *et al*., 2007), which permits the encapsidation of the HCV genomes and the formation of the nucleocapsids. Core and NS5A associate with each other and with LDs (Masaki, *et al*., 2008), which is crucial for the trafficking of viral replication complexes and RNA to LDs for virus production. NS3 is also recruited to LDs and it is predicted to be important for a stage in virus assembly after the interaction between core and NS5A at LD surfaces but

prior to assembly of core-containing particles (Ma, *et al*., 2008). Late assembly steps involve the acquisition of a lipid envelope and the incorporation of the E1/E2 glycoproteins into virions (Jones, *et al*., 2010). NS2 appears to confer infectivity to virions. They are thought to bridge glycoproteins and nascent particles together to form fully infectious HCV virions (Jones, *et al*., 2007). There is an expanding list of viral NSs that are predicted to engage in the process of assembly and release of HCV, such as p7 (without the precise knowledge at which stage p7 is involved) (Steinmann, *et al*., 2007) and NS4B, which is suggested to regulate virus production through its regulatory functions on other replicase components to increase delivery of genomes to packing sites (Jones, *et al*., 2009). Current models suggest that maturation and egress of HCV virions occur in concert with the production of very low density lipoproteins vLDL (Huang, *et al*., 2007). VLDL is a family of spherical particles that are produced only in liver to export triglyceride and cholesterol into plasma, and their production is a major function of hepatocytes (Gibbons, *et al*., 2004). During maturation, nascent viral particles become complexed with pre-vLDL; lipids, in the form of lumenal LDs; and other lipoprotein components, such as apoE (Boulant, *et al*., 2007) to generate lipoviroparticles (LVP) that are then released from the cell (Gibbons, *et al*., 2004). Knowledge about HCV assembly and release is increasing, with study in this area is still in its early stages, but rapidly progressing.

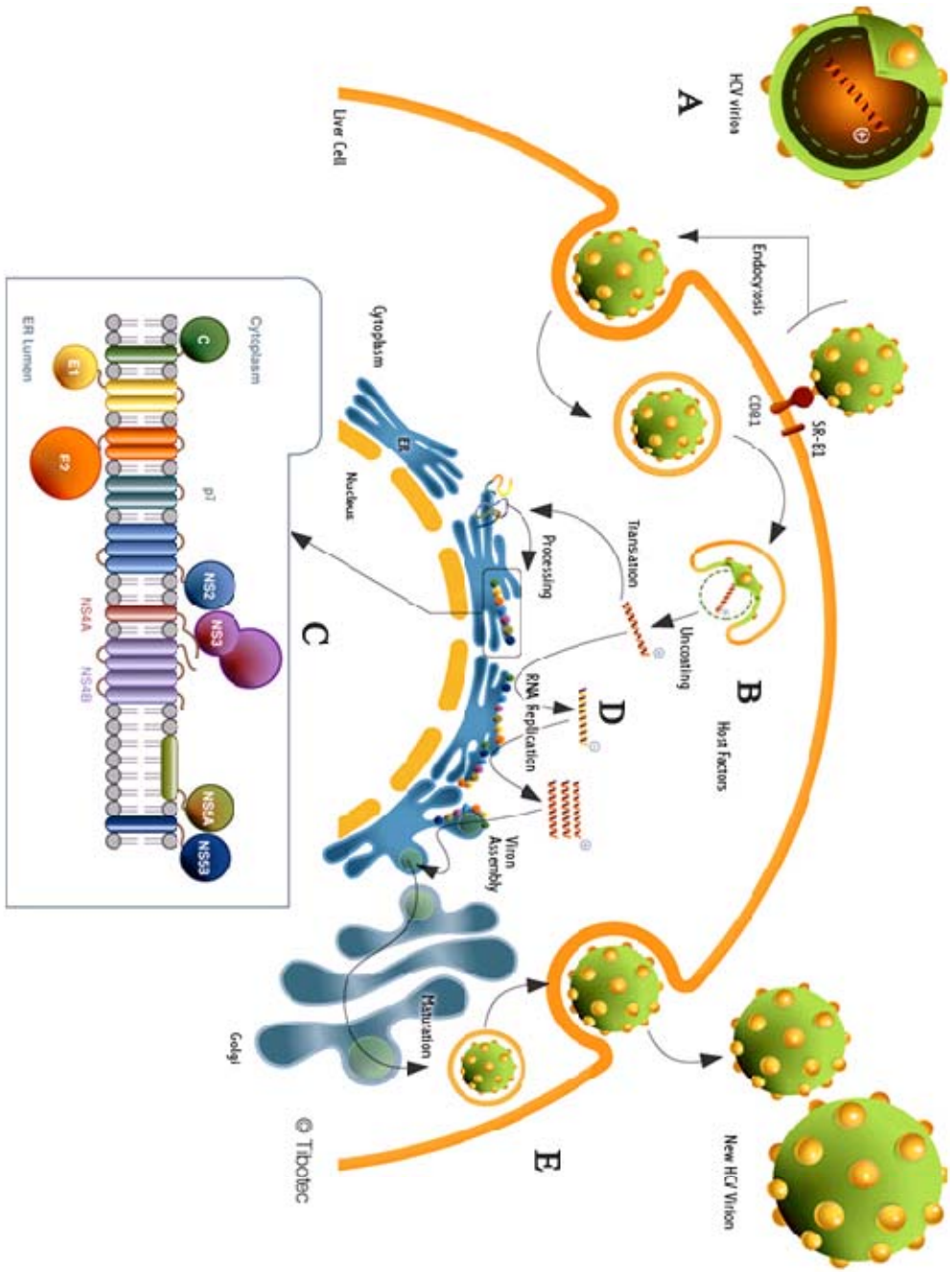


Figure 1-2 Lifecycle of HCV

(http://www.tibotec.com/content/backgrounders/www.tibotec.com/hcv_lifecycle.html).

(A): Virus binding by HCV receptors/co-receptors and internalization by endocytosis. (B): Cytoplasmic release from early endosomes and uncoating the viral RNA genome into cells. (C): IRES-mediated translation and polyprotein processing by cellular and viral proteases at ER

membrane. (D): HCV RNA replication occurs in the membranous web. Once there is adequate viral RNA transcriptase, + strand viral RNA serves as a template to generate – strand RNA intermediates and make the genetic materials for new viruses. (E): Packaging and assembly.

1.5 HCV Model Replication Systems----The HCV Replicon

The first breakthrough allowed detailed molecular studies of HCV replication, pathogenesis, and evolution in cell culture was the development of the HCV replicon system. The prototype subgenomic selectable replicon is a bicistronic RNA which encodes an authentic HCV 5' UTR, a neomycin phosphotransferase gene, a second IRES element from Encephalomyocarditis virus (EMCV) that drives translation of the non-structural proteins 3-5B, followed by the HCV 3'UTR (Lohmann, *et al*., 1999). Cells in which this RNA can replicate are selected by their ability to grow following selection with G418. In subsequent studies, full-length replicons and HCV genomes that replicate efficiently in a wide range of permissive host cells have been established. However, the latter stages of the virus life cycle including virion assembly and release were not amenable for detailed study until the discovery of HCV JFH-1. JFH-1 HCV (genotype 2a) was isolated from a Japanese patient with a rare case of fulminant hepatitis C and it was found to replicate in Huh-7 and other cell lines without the requirement for adaptive mutations, and produced infectious virions that could infect naïve Huh-7 cells (Kato, *et al*., 2003). Infectivity was enhanced by using highly permissive Huh-7 sub-lines (Zhong, *et al*., 2005) and chimaeric clones that consisted of the 5' and 3' UTRs, as well as NS3-5B regions of JFH-1 and the C terminus of NS2 protein of a different genotype 2a isolate designated J6 (Pietschmann, *et al*., 2006). HCVcc (for HCV cell culture) strain FL

J6/JFH-1 can develop long term infection in chimpanzees and in immunodeficient mice engrafted with human liver tissue. Viral inocula derived from these animal models were infectious for naive Huh-7 cells (Lindenbach, *et al*., 2006). Therefore, 17 years after the identification of HCV, the utility of the cell-culture-derived HCV (HCVcc) genetic system opened a new era for investigating the complete *in vitro* and *in vivo* HCV lifecycle, and analyzing the essential contribution of host cell factors to virus production. We are particularly interested in using these systems to study the interactions between the host miRNA pathway and HCV replication and translation.

1.6 MicroRNA and HCV Infection

1.6.1 MicroRNA Pathway

MicroRNAs (miRNAs) are non-coding small RNAs that regulate eukaryotic gene activity at the post-transcriptional level by inhibiting mRNA translation. More than 1000 miRNAs are expressed in mammals and are initially encoded as part of large precursor transcripts called primary miRNAs (pri-miRNA). Pri-miRNAs are processed into~60-70 nt hairpin precursor miRNAs (pre-miRNA) within the nucleus by a multiprotein complex called the Microprocessor, mainly composed of an RNase III enzyme, Drosha and the double-stranded RNA-binding domain (dsRBD) protein, DGCR8 (Denli, *et al*., 2004; Gregory, *et al*., 2004; Han, *et al*., 2004). Pre-miRNAs are exported to the cytoplasm by exportin-5 (Bohnsack, *et al*., 2004), where they are further processed by the cytoplasmic endonuclease III enzyme Dicer, with assistance by its binding partners, named human immunodeficiency virus 1 (HIV-1) transactivating response (TAR) RNA-binding protein

(TRBP) (Gatignol, *et.al.,* 1991) and protein activator of PKR (PACT) (Kok, *et al*., 2007; Lee, *et al*., 2006). The result is a ~22 nt long miRNA duplex bearing 2-nt overhangs at both 3' ends. Following miRNA processing, Dicer and TRBP function to integrate the newly synthesized miRNA into a trimeric protein complex containing Ago2, Dicer, and TRBP, a complex also termed the RNA-induced silencing complex (RISC), a ribonucleoprotein (RNP) complex. Once incorporated into RISC, the mature miRNA is unwound and guides RISC to complementary target sites in the 3' untranslated region (3' UTR) of messenger RNAs. On the basis of the degree of complementarities between the miRNA and the mRNA, RISC inhibits translation by an unknown mechanism. In some cases miRNA binding also alters mRNA stability (Gregory, *et al*., 2005; Bushati, *et al*., 2007) (Figure 1-3).

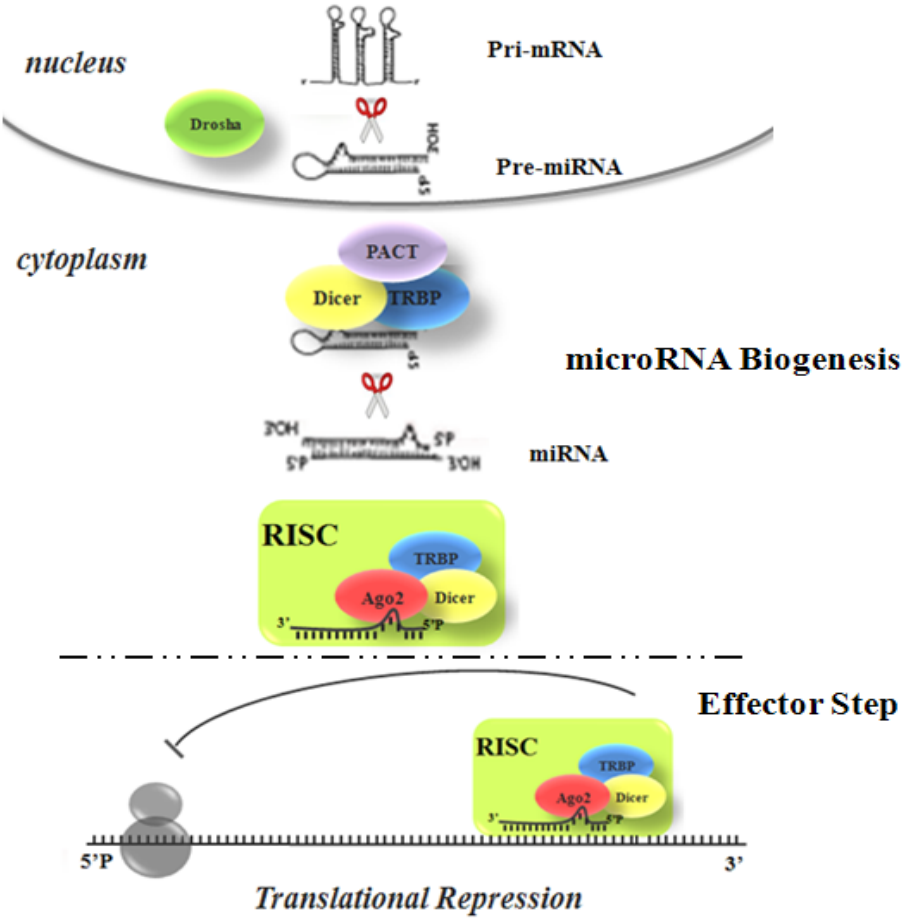


Figure 1-3: miRNA guided RNA interference in mammalian cells.

The endogenous mammalian miRNA pathway: begins with endogenous host primary mRNAs (pri-mRNAs) that are processed by the Drosha/DGCR8 complex to yield ~60-70 nt precursor miRNAs (pre-miRNAs). These hairpin-shaped pre-miRNAs are then exported to the cytoplasm by Exportin 5 and subsequently processed into ~22 nt mature miRNA duplex by Dicer and its partners TRBP and PACT. Dicer and TRBP then load the guide strand with lower stability in the 5' end is loaded into Ago2. MiRNAs can guide RISC to 3' UTR of complementary mRNAs. On the basis of the degree of homology between the miRNAs and the mRNAs, RISC can inhibit mRNAs function by inhibiting their translation.

It has been well established that miRNAs are involved in many basic physiologic processes including cell growth, tissue differentiation, embryonic development, cell proliferation and apoptosis. MiRNAs control various biological processes, including cell differentiation, cell proliferation, apoptosis, stress resistance, and fat metabolism (Hwang, *et al.,* 2006; Ambros *et al.,* 2003; Harfe, *et al.,* 2005). Cellular miRNAs are not only implicated in immune cell development and function (Lindsay, *et al.,* 2008), but are also directly implicated in defense against viral infections. In addition, some DNA viruses express miRNAs, such as herpesvirus, adenovirus, retrovirus and polyomavirus families (Cullen, *et al.,* 2006), and some are reported to suppress the innate immune response to promote virus growth (Lindsay, *et al.,* 2008). Certain miRNAs are expressed ubiquitously, whereas others exhibit a high level of specificity of expression, both according to tissue type, developmental stage, and cancer (Bushati, *et al*., 2007). MiR-122 is found specifically in mouse, woodchuck and human liver, human primary hepatocytes, and in cultured liver-derived cells, such as human hepatcellular carcinoma cell line 7 (Huh 7)

(Girard, *et al*., 2008; Jopling, *et al*., 2005). In human liver cells, miR-122 is identified to account for approximate 70% of the total liver miRNA population and expressed at a high level with more than 66,000 copies per cells (Jopling, *et al*., 2005; Girard, *et al*., 2008).

1.6.2 The Influence of miR-122 on HCV Replication

Seemingly intrigued by the tissue-specific nature of miR-122, Jopling and co-workers investigated its influence on HCV replication. They found that sequestering endogenous miR-122 in Huh-7 cells by antisense oligonucleotiedes (ASOs), which is able to completive bind to endogenous miR-122, caused a reduction of HCV RNA abundance at 48h post transfection, leading to their conclusion that miR-122 may stimulate HCV replication (Jopling, *et al*., 2006). By introducing mutations to the HCV 5' UTR and complementing miR-122 with compensatory mutations, the authors clearly demonstrated that the effects of miR-122 on HCV RNA abundance was through physical interactions of miR-122 with two tandem miR-122 binding sites located between stem-loops I and II of the highly conserved 5' UTR of HCV (Jopling, *et al*., 2008) (Figure 1-1).

In a recent study, Henke *et al*. (2008) identified that the interaction of miR-122 with the HCV 5' UTR also stimulated HCV translation at an early stage of HCV infection. MiR-122 was detected in the 48S translation initiation complexes with the HCV RNA. Moreover, the interaction between mutated miR-122 binding sites and the corresponding mutated miR-122 led to a strong stimulation in ribosome association in HeLa cells. The translation stimulation was observed even with an NS5B-polymerase defective genome. Supplement to Jopling's and Henke's work, Jangra *et al.* confirmed

the ability of miR-122 to promote the growth of infectious virus, including HCV replication and translation through direct interaction with both seed-sequence-binding sites (S1, S2) by studying virus infectivity using an infectious HCV strain (HJ3-5 virus). The S1 site appears to be more important for efficient replication than binding to the downstream S2 site, and the contribution of each binding site seems equally important for HCV translation, as shown by mutating the binding sites and supplementing with complementary mutant forms of miR-122. However, the positive regulation of miR-122 on HCV translation is insufficient to support the striking enhancement of HCV genome amplification and virus production. As an IRES mutant genome with a quantitatively equivalent defect in translation generated much better viral production than that of double-binding-site mutant genome. Thus, their study predicted that miR-122 is likely to act to stimulate both replication and translation (Jangra, *et al*., 2010).

The effect of miR-122 to augment the replication and translation of HCV is unusual. This is the only case in which a miRNA binds to RNA genome and promotes RNA polymerization. The only similar function might be in the amplification of siRNAs in plants and in worms and it has always intrigued me that small RNAs might have interesting influences on RNA directed RNA polymerases.

Stimulation of translation of an mRNA following miRNA binding is rare but not unique to HCV. MiR-10a has a similar function in the promotion of translation by binding the conserved 5' terminal oligopyrimidine tract (5'TOP) of the ribosomal protein (RP) mRNAs (Orom, *et al*., 2008). Under conditions of amino acid starvation, miR-10a functions to repress translation of RP mRNAs, however, following anisomycin treatment or overexpression of RAS, miR-10a switches to a role into translational induction. 5’TOP

motif comprises the core of the translational cis-regulatory element of the mRNAs involved in translation and ribosome biogenesis and confers sensitivity to mitogens and nutrients in eukaryotes (Meyuhas, *et al*., 2000). Therefore, the translation control of TOP mRNAs is important for the biosynthesis of the translational machinery and cell growth. However, it is interesting to note that miR-10a was found to be capable of both translation repression via interaction with binding sites in the 3'UTR and translation enhancement through associating with 5'UTR of different groups of mRNAs. Similarly, miR-122 can also induce translational repression of an mRNA construct bearing miR-122 binding sites in its 3'UTR (Jopling *et al*., 2008). Therefore, the same miRNA may behave differently depending on the binding site positions and also cellular growth conditions.

MiR-122 functions as a key regulator of lipid and cholesterol biosynthesis in the liver. Sequestration of endogenous miR-122 by locked-nucleic-acid-modified oligonucleotide (LNA-antimiR) resulted in reduced mevalonate pathway activity, suppression of liver and plasma cholesterol levels, as well as reduced fat accumulation in the livers of mice and non-human primates (Elmen, *et al*., 2008). Furthermore, evidence indicates that miR-122 is essential for maintaining liver cell identity and serves as a diagnostic and prognostic marker; its inhibition could increase the risk of developing hepatocellular carcinoma (Bai, *et al*., 2009). Since cholesterol and lipoproteins are integral components of virion structure, and important for viral circulation and lifecycle (Andre, *et al*., 2002; Kapadia, *et al*., 2007), it is suggested that miR-122 could also be indirectly linked to regulate viral entry, assembly or egress of infectious virus. It will become an invaluable target to expand our knowledge in the pathophysiology of diverse liver diseases.

Lanford and colleagues recently found that sequestering miR-122 in the liver of chimpanzees by intravenous injection of LNA SPC3649, resulted in a dramatic reduction in serum HCV without yielding emergent viral resistance and side effects to host liver functions (Lanford, *et al*., 2010). Their findings pointed the way to study alternative cellular targets for anti-HCV therapies; in particular, targeting miR-122 may become a potential novel antiviral approach to restrain HCV infection. A better understanding of the mechanisms responsible for miR-122 modulating HCV infection and its dependence on the critical host factors will greatly contribute to the development of innovative and more effective antiviral therapeutics. Motivated by these scenarios, we started our investigations with two essential miRISC proteins responsible for miRNA biogenesis in the miRNA pathway, Dicer and TRBP.

1.7 Human Dicer

Dicer is a cytoplasmic RNase III family protein found in most eukaryotes. Mammalian genomes encode only one Dicer protein. Human Dicer is a large (~220 kDa) multi-domain protein with an N-terminal DExH/DEAH box RNA helicase domain, a domain of unknown function (DUF283), a Piwi Argonaute Zwille (PAZ) domain, two RNaseIII domains (RIIIa and RIIIb), and a C-terminal double-stranded RNA binding domain (dsRBD) (Murphy, *et al*., 2008; Murchison, *et al*., 2004). The DExH/DEAHbox family of ATP-dependent RNA helicase plays a central and essential role in the miRNA machinery, including unwinding miRNA precursors. In addition, the Dicer helicase has been suggested to act as an “RNPase”, remodeling the processing steps and the interaction between miRNAs and other RNAs entering the miRNA pathway and

ribonucleoprotein complexes (RNPs) (Schwer, *et al*., 2001). So far, the dsRNase activity of either recombinant or endogenous human Dicer exhibits no ATP requirement *in vitro* (Zhang, *et al*., 2002; Provost, *et al*., 2002); however, this cannot preclude the existence of a distinct ATP-dependent catalytic activity mediated by additional factors in the RNAi pathway *in vivo.*

Dicer recognizes its pre-miRNA substrates in a polar manner with the RNase IIIa domain always processing the protruding 3'-OH-bearing RNA strand, and RNase IIIb cleaving the opposite 5'-phosphate-containing strand to generate products with 2 nt 3' overhangs (Zhang, *et al*., 2004). The 2 nt 3' overhangs are recognized by the PAZ domain. PAZ is a highly conserved domain unique to Dicer and Ago proteins. PAZ in Dicer has a particular specificity for miRNA/siRNA with 2 nt 3' overhang structure characteristic of RNase III processing, which suggests that Dicer plays an essential role in the recognition and selection of pre-miRNA substrates pre-processed by Drosha (Murchison, *et al*., 2004). Dicer is also involved in loading of miRNAs into the RISC complex (Chendrimada, *et al.,* 2005; Doi, *et al.,* 2003) and interestingly, *in vitro* assembly of RISC with pre-miRNA was more efficient than with pre-cleaved miRNA duplex, indicating that the miRNA/siRNA biogenesis and RISC loading steps are tightly coupled (Gregory, *et al*., 2005). The mechanism of dsRNA processing and RISC loading by human Dicer has been most extensively investigated; however, possible functions of Dicer in the effector steps of posttranscriptional regulation are not fully elucidated.

Dicer activities in many cases are crucial to the cell. General knockout of Dicer1 in the mouse causes morphologic abnormalities and stunted growth in embryonic day (E) 7.5 embryos and embryonic lethality by day 11.5 (Bernstein, *et al*., 2003). In order to

study the effects of Dicer knock-out in particular tissues, conditional knockout of Dicer has been explored by developing Dicer alleles with the second RNAse III domain flanked by loxP sites (Dicer fl/fl) to facilitate conditional knockdown (cKO) of Dicerfl/fl via tissue-specific recombination by Cre recombinase (Bernstein, *et al*., 2003). Disrupting Dicer was found to compromise formation of miRNAs and was found to influence vertebrate development (Bernstein, *et al*., 2003; Giraldez, *et al*., 200), alter cell morphology (Harris, *et al*., 2006), increase apoptosis (Asada, *et al*., 2008), decrease cell proliferation (Murchison, *et al*., 2005), and inhibit differentiation (Kanellopoulou, *et al*., 2005). Other processes controlled by Dicer related miRNA activities which were disrupted in conditional Dicer knockouts were the decreased formation of heterochromatic structures (Fukagawa, *et al*., 2004) and reduced centromeric silencing (White, *et al*., 2004). Dicer may play an important role in the development of cancer through epigenetic regulation of as yet unknown genes. In most cases, knockout of Dicer reduced cell proliferation and up regulated apoptosis, for example, knockdown of Dicer was shown to inhibit human breast carcinoma cell growth via G1 arrest and up-regulation of cell cycle-dependent kinase inhibitor (CKI) p21 and p27 (Bu, *et al*., 2009). However, a few exceptions exit. Inhibition of Dicer was reported to enhance tumor proliferation and invasion via activated p-Akt pathway and enhanced expression of the cell cycle associating molecules, cyclin A and PCNA, as well as MMP-2 and MMP-9 (Han, *et al*., 2010). In addition, conditional depletion of Dicer from murine Schwann Cells was found to increase their proliferation while blocking myelination (Bremer, *et al*., 2010; Pereira, *et al*., 2010). Also, dicer knockdown in Huh 7.5 cells caused no significant effect on cell proliferation (Randall, *et al*., 2007). In general, it appears that Dicer is essential for

efficient cell growth, but in certain cases Dicer knockdown or knockout can have a neutral or even positive effect on cell growth.

1.8 Human TRBP

TRBP1 and TRBP2 are two isoforms transcribed by the human tarbp2 gene. The two mRNAs are initiated from adjacent promoters that transcript alternative first exons, which are spliced into common downstream exons. As a consequence, in comparison to TRBP1, TRBP2 has 21 additional amino acids at its N-terminus (Bannwarth, *et al*., 2001). TRBP2 has two double-stranded RNA binding domains, the second one containing a KR-helix motif that mediates dsRNA binding (Daviet, *et al*., 2000). A third basic domain, Medipal domain in the C-terminal end of TRBP mediates protein-protein interactions (Laraki, *et al*., 2008).

The TRBPs were identified through their ability to bind the HIV-1 transactivating response (TAR) RNA (Daher, *et al*., 2001) and stimulate of the expression of the HIV long terminal repeat (LTR) in human and murine cells (Gatignol, *et al*., 1991).

In mammalian cells, TRBP (~45 KDa) was found to be a protein binding partner of Dicer (Rossi, *et al.,* 2005) through the C-terminal binding site of TRBP (Daniels, *et al*., 2009). TRBP is required for RNAi function mediated by both siRNAs and miRNAs. Knocking down either Dicer or TRBP resulted in the destabilization of both proteins and a substantial decrease in the accumulation of mature miRNAs (Chendrimada, *et al*., 2005). TRBP is proposed to act as a biosensor in strand selectivity that ensures proper loading of the 3' end of the guide strand onto the Ago2 (Castanotto, *et al.,* 2007). In the

absence of TRBP, the Ago2-Dicer complex disassociates entirely under dilution conditions used for EM grid preparation. This observation suggests that TRBP increases the affinity of Ago2 for Dicer, and that TRBP facilitates the association of Dicer and Ago2 in the process of miRNA loading into RISC (Wang, *et al*., 2009; Chendrimada, *et al*., 2005). Furthermore, Dicer, TRBP and Ago2 are necessary and sufficient for *in vitro* reconstitution of the RNAi activity (MacRae, *et al.,* 2008)

TRBP was found to increase HIV protein expression and replication by directly binding to the interferon-induced dsRNA-activated protein kinase PKR through its dsRBDs and blocking the inhibitory effects of PKR on HIV (Ong, *et al*., 2005). TRBP has a direct effect on stimulating the translation of TAR containing structured RNA in $PKR^{-/-}$ MEF, which is independent of its ability to inhibit the translational block imposed by PKR (Dorin, *et al*., 2003).

TRBP functions as a translational regulator during mouse spermatogenesis and has a physiological role in growth control during development (Zhong, *et al*., 1999). Overexpression of TRBP in NIH3T3 cells caused enhanced cell growth and transformed phenotypes, such as anchorage-independent cell growth and tumor development in mouse xenografts (Benkirane, *et al.,* 1997). A tumor suppressor named merlin was shown to efficiently inhibit these oncogenic activities of TRBP (Lee, *et al*., 2004).

1.9 Hypothesis and Objectives

Previous research on identifying cellular cofactors involved in the host miRNA pathways that affect HCV infection and replication inspired my thesis work. Randall *et al*. (2007) demonstrated that siRNA-mediated knockdown of Drosha, DGCR8, Dicer, or each of the four Argonaute proteins significantly diminished the accumulation of viral RNA and infectious virus production in HCV-infected cells. In addition, work in our laboratory on the effects of Ago knock-down on HCV supported Randall's work. However, the mechanism by which the miRNA pathway is involved in HCV infection remains unsolved. Randall *et al*. hypothesized that the inhibition of HCV by siRNA targeting Dicer may be due to Dicer's function in miR-122 biogenesis; however, they did not further investigate whether endogenous miR-122 levels were decreased by Dicer knockdown.

In this study, we aimed to provide evidence of the significance of RISC proteins in modulating HCV infection and deepen our understanding of the mechanism. Our laboratory supports a model in which the mechanism of HCV replication augmented by miR-122 involves the association of miR-122 with a protein complex, similar to the RISC complex. Since Dicer and TRBP are two essential RISC proteins that may play a role in downstream RISC function through the association with Agonaute proteins, we considered the possibility that Dicer and TRBP are required for both miR-122 biogenesis and miR-122 mediated HCV replication, most likely through functions in miRNA loading into Ago2 and RISC.

My research was motivated by two hypotheses:

I. Knock-down of Dicer and TRBP decrease HCV replication and translation.

II. Dicer and TRBP are not only responsible for miR-122 biogenesis, but also modulate HCV replication through their roles in RISC assembly step of RNA interference.

To test these hypotheses, I established the following objectives:

I. Design siRNAs to knock down Dicer and TRBP and observe the effects on full length J6/JFH-1 HCV replication and translation in Huh7.5 cells.

II. Determine specific roles of Dicer and TRBP in HCV replication by analyzing endogenous miR-122 changes following Dicer and TRBP knockdown.

III. Design an HCV replication assay independent of wild type miR-122 by introducing mutations to seed sequence binding sites of 5'UTR, and characterize whether Dicer and TRBP have direct roles in mutant HCV replication complemented by miR-122 with complementary mutations, such as their roles in RISC assembly step of the RNAi machinery.

2.0 MATERIALS AND METHODS

2.1 Viruses:

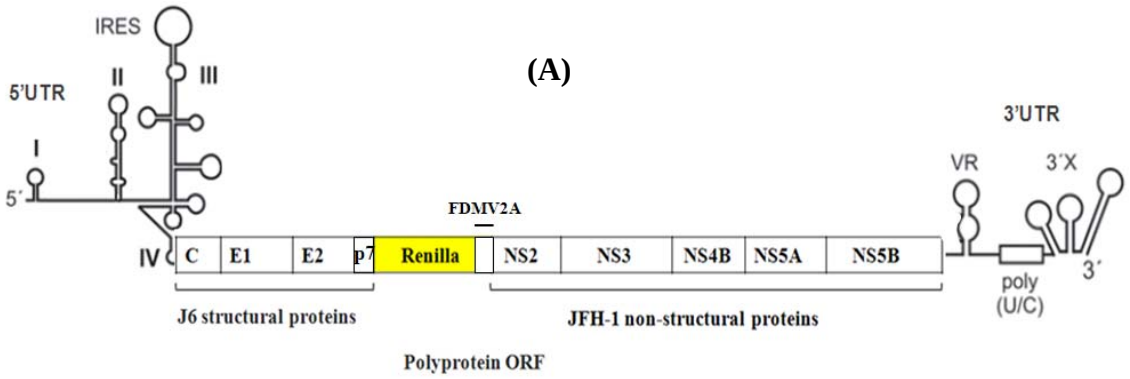
FL-J6/JFH-1-RLuc-WT is a full length-genotype 2a sequence that is capable of replication and virus particle production in cell culture. It is a chimeric virus RNA containing a 5' untranslated region (5' UTR) from the genotype 2a JFH-1 isolate, structural proteins core through NS2 from the genotype 2a J6 isolate and non-structural proteins through 3'UTR from the genotype 2a JFH-1 isolate. It also contains an inserted Renilla Luciferase (RLuc) reporter gene between p7 and NS2. RLuc is cleaved from the polyprotein at p7 downstream and foot-and-mouth disease virus (FMDV) 2A peptide cleavage site upstream of NS2 (Jones, *et al.,* 2007) (Figure 2-1A).

FL-J6/JFH-1-RLuc-GNN: is a non-replicative mutant of HCV J6/JFH-1. It is identical to the replicative wild-type genome, but contains a point mutation which abolishes RNA polymerase activity of NS5B (Tomoko D. *et al.,* 2004) (Figure 2-1B).

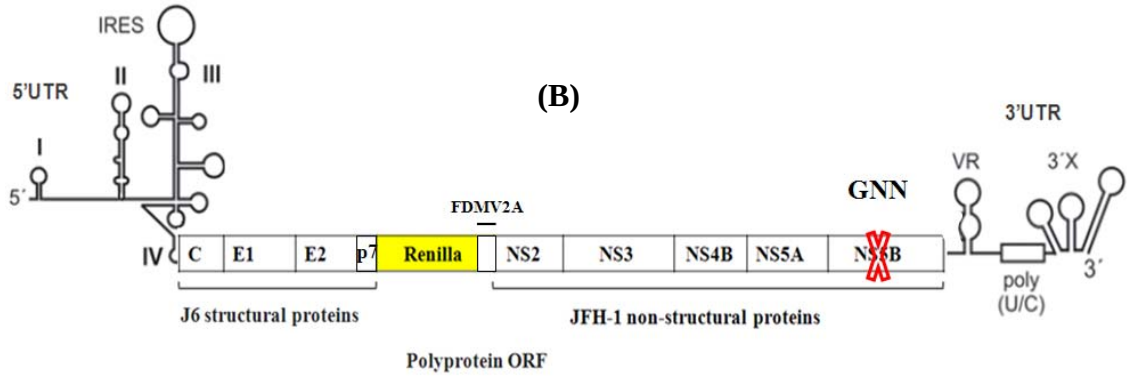
FL-J6/JFH-1-RLuc-m3-4: nucleotides at positions 3 and 4 of each miR-122 binding site were mutated to their complements (i.e., U26C to A27G and U41C to A42G), such that the mutant sites are not predicted to bind wild-type miR-122 (wt miR-122). Instead, they should bind to mutant miR-122p3-4, which was designed to restore base pairing to the viral RNA-miRNA pair (Jopling. *et al.,* 2008) (Figure 2-1C).

Figure 2-1: Schematic constructions of chimeric full length HCV genome and derivates: (A). FL-J6/JFH-1-RLuc-WT (B). FL-J6/JFH-1-RLuc-GNN (C). FL-J6/JFH-1-RLuc-m3-4

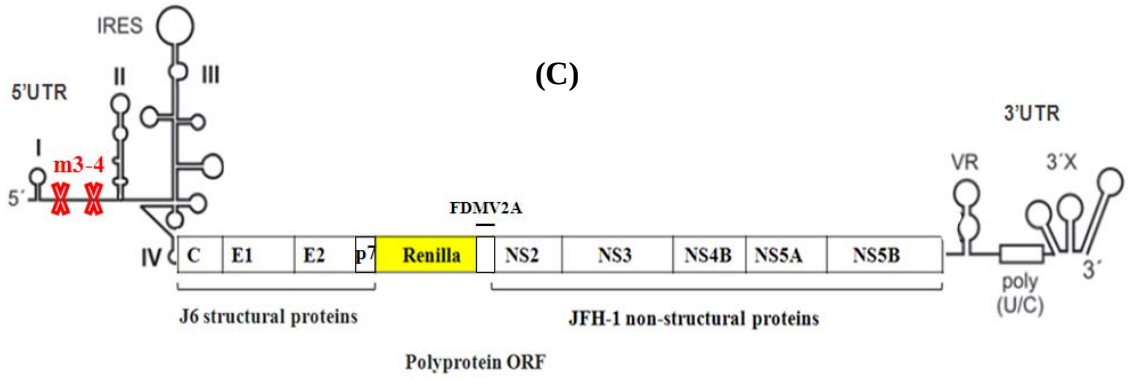
(A)


IRES
5'UTR
I
II
III
IV
5´
3'UTR
VR
3´X
3´
poly
(U/C)
FDMV2A
C
E1
E2
p7
Renilla
NS2
NS3
NS4B
NS5A
NS5B
J6 structural proteins
JFH-1 non-structural proteins
Polyprotein ORF

(B)


IRES
5'UTR
I
II
III
IV
5´
3'UTR
VR
3´X
3´
poly
(U/C)
GNN
FDMV2A
C
E1
E2
p7
Renilla
NS2
NS3
NS4B
NS5A
NS5B
J6 structural proteins
JFH-1 non-structural proteins
Polyprotein ORF

(C)


IRES
5'UTR
I
II
III
IV
5´
m3-4
3'UTR
VR
3´X
3´
poly
(U/C)
FDMV2A
C
E1
E2
p7
Renilla
NS2
NS3
NS4B
NS5A
NS5B
J6 structural proteins
JFH-1 non-structural proteins
Polyprotein ORF

2.2 Cell Culture:

Huh 7.5 cells are a subline derived from Huh7 human hepatoma cells and are highly permissive for HCV replication (Blight KJ, *et al.,* 2002). Huh7.5 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Burlington, ON Canada) supplemented with 10% fetal bovine serum (FBS, Multicell, Wisent Inc., St. Bruno, QC, Canada), 1% Penicillin Streptomycin (10,000 units/ml Penicillin, 10,000 µg/ml Streptomycin) (Invitrogen, Burlington, ON Canada) and 1% nonessential amino acids (100x, Multicell, Wisent Inc.; St. Bruno, QC, Canada) at 37ºC in the presence of 5% CO_2.

2.3 siRNAs/microRNAs

Table 2-1. The sequences of siRNAs/miRNAs used in this study.

siRNAs/miRNAs	sequences (5'---3')	Company
control siRNA 6367	AAGACACUGAGACACCAAUUGAC (Wilson J. *et al.,* 2003)	Invitrogen
siDicer	GGC UUA UAU CAG UAG CAA Utt	Ambion
siTRBP	CAC CGC AAA GAA UUC ACC AUG ACC U	Invitrogen
Control miRNA	miRIDIAN microRNA Mimic Negative Control Sequence is not available	Thermo Scientic Dharmacon
wt miR-122	UGG AGU GUG ACA AUG GUG UUU GU	Thermo Scientic Dharmacon
miR-122p3-4	UGC UGU GUG ACA AUG GUG UUU GU	Thermo Scientic Dharmacon
miR-122*	AAA CGC CAU UAU CAC ACU AA A UA	Thermo Scientic Dharmacon

Mature miRNA duplexes were annealed with equimolar amounts of either wild type or p3-4 forms of miR-122 with a common complementary miRNA strand miR-122*.

2.4. In Vitro Transcription of HCV RNA

2.4.1 Preparation of viral RNA from full length HCV plasmid constructs

5 µg of plasmid encoding full length chimeric HCV genome was digested with 2µl XbaI (New England Biolabs, ON Canada) combined with 1 x BSA and 1 x NEB 4 (New England Biolabs, ON Canada). The reaction mixture was brought to the final volume of 45 µl by ddH_2O and incubated at 37° C for 1 hour. Addition of another 1.5 µl of XbaI for another hour gave more efficient digestion. 5' overhangs of linearized DNA were removed by adding 1 µl of mung bean nuclease (New England Biolabs, ON Canada) at 37° C for 1 hour.

2.4.2 Phenol and Chloroform Extraction of Linearized DNA

Linearized DNA was extracted by using 25 µl each of tris-saturated phenol (Invitrogen, *Burlington*, ON Canada) and chloroform (Sigma; Oakville, ON, Canada) to make an equal volume to the sample. The DNA was precipitated by adding 5 µl of 3 M sodium acetate combined with 100 µl of 100% ethanol, and chilled at -20° C for a minimum of 2 h. DNA pellets were centrifuged at 14,000 x rpm for 10 min and resuspended in 10 µl ddH_2O.

2.4.3 *In Vitro* Transcription of HCV RNA

In vitro transcription of HCV RNA was performed using MEGAscript® T7 Kit (Ambion; Streetsville, ON, Canada) following the supplied protocol.

Table 2-2. MEGAscript®T7 In vitro Transcription Reaction Assembly mix:

Component	Amount
ATP solution	2 µl
CTP solution	2 µl
GTP solution	2 µl
UTP solution	2 µl
10X Reaction Buffer	2 µl
linear template DNA plasmid template	1 µg
Enzyme Mix	2 µl
Nuclease-free Water	To 20 µl

The transcription reaction was incubated overnight at 37° C in an air incubator. On the following day, the reaction was treated with 1 µl of TURBO DNAse at 37° C for 20 min to remove the template DNA and the reaction was then terminated by adding 115 µl Nuclease free Water and 15 µl Ammonium Acetate Stop Solution.

2.4.4 Phenol and Chloroform Extraction and RNA Precipitation

HCV RNA was extracted with equal volumes of phenol/chloroform (75 µl), and then extracted once with an additional 150 µl of chloroform. The RNA containing

aqueous phase was collected and the RNA precipitated by adding 150 µl isopropanol and chilling for at least 2 hours at -20° C. RNA pellets were centrifuged and resuspended in 50 µl DEPC-H_2O, and RNA concentration was determined by using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purified total RNA samples were stored at -80º C.

2.5 Transient HCV Replication Assay

Huh7.5 cells were washed with ice-cold Dulbecco's DPBS (Multicell, Wisent Inc.; St. Bruno, QC, Canada), trypsinized by 2 µl trypsin-EDTA (0.05 % trypsin, 0.53 mM EDTA) (Multicell, Wisent Inc.; St. Bruno, QC, Canada), and resuspended in complete growth medium. Cells were pelleted by centrifugation (1000 x *rpm* for 5 min at 4°C), then washed twice with ice-cold DPBS, and resuspended in ice-cold DPBS at 1 x 10^7 cells/ml. 400 µl cell suspensions were mixed with 60 nM siRNAs (control siRNA, siDicer and siTRBP) and placed into 4 mm gap electroporation cuvettes (VWR, Edmonton, AB, Canada). Electroporation was performed by using Gene Pulser X cell™ (Bio-RAD, Mississauga, ON, Canada) with settings: 270 v, 950 µF, ∞ Ω, 4mm cuvette. Following the electroporation, cells were immediately resuspended in 8 ml of complete growth medium. Approximate 6 x 10^6 electroporated cells were plated into 150 x 15 mm tissue culture dishes (Sarstedt, Inc. Newton NC, USA) and grown at 37° C for 3 days prior to the second electroporation. The second electroporation was performed on day 3 post electroporation (p.e.) using the same procedure as described above, but cells were co-electroporated with 5 µg of FL-J6/JFH-1-RLuc-WT (Figure 2-1) or FL-J6/JFH-1-RLuc-GNN RNA (Figure 2-2) transcripts and 60 nM of the same siRNA used in the first electroporation. Cells were resuspended in 8 mL of media and plated as required for

subsequent assays, and incubated at 37° C prior to harvest. 1.2 x 10^5 cells were plated for luciferase assay and cell lysates were collected at 1 h and 72 h post second electroporation (p.s.e.). 9.6 x 10^6 cells were plated for RNA analysis at 72 h p.s.e. 4.8 x 10^3 cells were plated and cell viability was measured by WST-1 Assay at 72 h p.s.e. 2.4 x 10^5 cells were plated and harvested at 72 h p.s.e for protein analysis.

2.6 HCV Translation Assay

Similar to the method used in the transient replication assay, Cells were prepared and electroporated with siRNAs to knockdown the desired genes (control siRNA, siDicer and siTRBP). Three days later the cells were electroporated a second time with 5 μg FL-J6/JFH-1-RLuc-GNN RNA (Figure 2-2), and 1μg capped Firefly Luciferase (FLuc) reporter mRNAs as endogenous control. 1.2 x 10^5 cells were plated for luciferase assay and cell lysates were collected at 3.5 h, 4 h, and 4.5 h p.s.e. 9.6 x 10^6 cells were plated for qRT-PCR analysis and collected at 4 h p.s.e. 4.8 x 10^3 cells were plated and cell viability was measured by WST-1 Assay at 24 h p.s.e.

2.7 HCV Transient Replication Assay for miR-122p3-4 Rescue

Cells were prepared for electroporation as described in section 2.5 and electroporated with 5 μg FL-J6/JFH-1-RLuc-m3-4 (Figure 2-3), or 5 μg FL-J6/JFH-1-RLuc-GNN RNAs, together with 60 nM siRNAs (control siRNA, siDicer and siTRBP) and 40 nM miRNAs (micontrol or miR-122 duplex) were co-electroporated into huh 7.5 cells. 1.2 x 10^5 cells were plated for luciferase assay and cell lysates were collected at 1 h

and 72 h post electroporation (p.e.). 4.8 x 10^3 cells were plated and cell viability was measured by WST-1 Assay at 72 h p.e.

2.8 WST-1 Assay

To test cell viability, 4.8 x 10^3 HCV-infected huh 7.5 cells were seeded into 96-well plates with 100 µl culture medium. At 72h p.s.e., the culture medium was changed with 110 µl/well fresh medium containing 10µl WST-1 (Roche Diagnostics, Laval, Quebec, Canada) and cells were incubated for another 4 h at 37 ºC, 5% CO_2. A triplicate of blank wells with the same volume of culture medium and WST-1 were set as a background control. Standard curves were generated by 2 - fold dilutions of 9.6 x 10^3 unelectroporated cells. The absorbance of the samples against a background control as blank was measured using a microplate (ELISA) reader Spectramax 340PC (Molecular Devices) with the wavelengths 450 / 650 nm. Cell numbers directly correlating with absorbance were calculated from the standard curve.

2.9 Western Blotting

Dicer and TRBP protein expression levels were analyzed by SDS-PAGE. 2.4 x 10^5 cells were plated and total Cell lysates were collected at 72h p.s.e. in 1 x SDS lysis buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.7, 30% glycerol, 0.002% bromophenol blue, 2% β-mercaptoethanol) and 1 M Dithiothreitol (Sigma, Oakville, ON, Canada) before being denatured at 96º C. Whole cell extracts were electrophoresed on an SDS-PAGE gel consisting of a 4% stacking gel and 12% resolving gel for TRBP and 6% for Dicer (due to different molecular weights of proteins). For electrophoresis, 2 µl of Bench Mark™ prestained protein ladder or Novex® sharp pre-stained protein standards (Invitrogen,

Burlington, ON Canada) and 25 µl of each sample were loaded on the SDS-PAGE gel and run in 1 x SDS running buffer (2.5 mM Tris-HCl, 192 mM glycine and 0.1% SDS, pH 8.3) at 150 V for 1 h (until loading dye ran off the gel). The separated proteins in the SDS-PAGE gel were transferred to a Hybond C+ nitrocellulose membrane (Amersham Pharmacia Biotech, *Québec,* Canada) for 90 min at 400 mA in 1 x transfer buffer (28.8 g glycine, 6.04 g Tris base, 200 ml menthol, 1.6 L ddH_2O). The membranes were blocked for 1 h at Room Temperature (RT) with 3% BSA in 1 x D-PBS, and incubated overnight at 4º C with primary Dicer 14601 monoclonal antibody (Abcam Cambridge, MA, USA) diluted 1/100, or TRBP 42018 polyclonal antibody (Abcam Cambridge, MA, USA) diluted 1/500, together with β actin 6276 monoclonal antibody (Abcam Cambridge, MA, USA) at a 1/1000 dilution. The next day, the membranes were washed in PBS-A 3 times and treated with secondary Odyssey goat anti-Rabbit IRDye@680 for TRBP1, Odyssey Goat anti-Mouse IRDye@880 (*Li-Cor* Biotech, Lincoln, NE, USA) for Dicer and β-actin diluted 1/10,000 in 3 % BSA. The membranes were then incubated for 1 h at RT and washed three times in 1 x D-PBS in a dark environment. Protein bands were visualized and quantified by Odyssey® Infrared Imaging System (*Li-Cor* Biotech, Lincoln, NE, USA) according to the manufacturer's protocol.

2.10 Luciferase Assay:

For HCV transient replication assay, 1.2 x 10^5 cells were plated. Infected cells lysates were collected at 1 h and 72 h p.s.e. and washed twice with 1 x D-PBS and lysed by adding 100 µl 1 x Renilla luciferase lysis buffer (Promega Biosciences; Nepean, ON, Canada) and rocked gently at room temperature (RT) for 15 min. Renilla luciferase

activity was measured using the GLOMAX luminometer (Promega Biosciences; Nepean, ON, Canada) by mixing 10 µl cell lysates with 50 µl 1 x Renilla luciferase assay buffer (Promega Biosciences; Nepean, ON, Canada).

For HCV transient translation assay, washed cells were lysed in 100µl 1 x passive lysis buffer (Promega Biosciences; Nepean, ON, Canada). FLuc activity was determined by mixing 10 µl cell lysates with 50 µl Dual luciferase assay buffer (Promega Biosciences; Nepean, ON, Canada), and measuring luminescence on the GLOMAX luminometer, and then adding 50 µl Stop & Glow® Reagent (Promega Biosciences; Nepean, ON, Canada) to quench FLuc activity and activate Renilla Luciferase for a second measurement on the GLOMAX luminometer.

2.11 RNA Analysis

2.11.1 Total RNA Isolation

9.6 x 10^6 electroporated cells were seeded for RNA analysis. For quantitating viral RNA, total RNA was extracted from cells with 1 ml Trizol reagent (Invitrogen, Burlington, ON Canada) at 72h p.s.e. according to the manufacture's protocol. Cell lysates were incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of Trizol was added. The samples were vigorously shaken by hand for 15 s and incubated at RT for 2-3 min. The samples were then centrifuged at 12,000 x g for 15 min at 4° C. After centrifugation, the colorless upper aqueous phase was extracted and transferred to fresh tubes. RNA in the aqueous phase was precipitated by mixing with 0.5 ml isopropyl alcohol (isopropanol) per 1 ml of TRI_{ZOL} reagent used for the initial homogenization. The RNA samples were vigorously shaken by hand for 15 s and incubated at RT for 10 min, following by

centrifuging at 12,000 x g for 15 min at 4° C. Following centrifugation, the supernatant was removed and RNA pellet was washed with l ml 75% ethanol and centrifuged at 7,500 x g for 5 min at 4° C. At the end of the procedure, the RNA pellet was briefly air-dried, resuspended in an approporiate volume of DEPC-H_2O and quantified using a NanoDrop ND-1000 Spectrophotometer. Purified total RNA samples were stored at -80º C for further analysis.

2.11.2 Quantitative Reverse Transcriptase PCR (qRT-PCR).

2. 11.2.1 TaqMan Gene Expression Assay

First-strand cDNA synthesis was performed using iScript™ Select cDNA Synthesis Kit (Bio-RAD, Mississauga, ON, Canada). A volume of 1 µg total RNA was combined with 4 µl of 5 × iScript select reaction mix, 2 µl of random primer, 1 µl of iScript reverse transcriptase, and RNase-free water to a final volume of 20 µl. For random-primed cDNA reaction, the reaction mixture was incubated at 25° C for 5 min, 42° C for 30 min, and the reverse transcriptase was heat-inactivated at 85° C for 5 min. The cDNA products were stored at -20° C for further analysis.

Relative amounts of Dicer or TRBP to human GAPDH mRNAs were quantified by using TaqMan Gene Expression Assays (*Applied Biosystems, Carlsbad, CA,* USA) according to manufacturer's protocol. Gene specific fluorescent probes for Dicer (Hs00229023_ml 20 x mix), TRBP1 (Hs00366328_ml 20 x mix), and GAPDH (FAM-MGB 4352934-0803022) were commercially purchased from Applied Biosystems.

Table 2-3. TaqMan Gene Expression Assay reaction mix:

Component	**Volume (µl) / Reaction**
TaqMan Gene Expression Assay (20x)	1.0
cDNA template +RNase free H_2O	9.0
TaqMan Universal PCR Master Mix (2x)	10.0
Total Volume:	20.0

The qRT-PCR step was performed in 96-well PCR plates in triplicate by using CFX 96 ^{TM} system (Bio-RAD, Mississauga, ON, Canada).

Table 2-4. qPCR thermal cycling conditions for TaqMan Gene Expression Assay:

Step	**AmpliTaq Gold Enzyme Activation**	**PCR**	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/Extend
Time	10 min	15 s	1 min
Temp	95° C	95° C	60° C

2.11.2.2 Eva Green Gene Expression Assay

For studying HCV transient translation, relative RNA amounts of HCV to firefly luciferase reporter mRNA were quantified by Eva Green gene expression assay. First-strand cDNA synthesis was performed using iScript™ Select cDNA Synthesis Kit (Bio-RAD, Mississauga, ON, Canada) with JFH-1 FL/SGR primer: 5' CGGTGAACCAACTGGATAAG 3' to reverse transcribe the whole HCV genome from 3'UTR, and firefly luciferase primer: 5' TTTGGACTTTCCGCCCTTCTTG 3'.

Table 2-5. iScript™ Select cDNA RT reaction master mix for specific primers:

Component	**Volume (µl) / Reaction**
5x iScript select reaction mix	4 µl
FL-SGR JFH-1 primer	500 nM
Firefly luciferase primer	500 nM
GSP enhancer solution	2 µl
Total RNA (1 µg)	Variable
iScript reverse transcriptase	1 µl
Nuclease-free water	Variable
Total Volume:	20.0

For gene-specific primers, reverse transcription was incubated at 42° C for 60 min, and the reverse transcriptase was heat-inactivated at 85° C for 5 min. The cDNA products were stored at -20° C for further analysis.

Relative amounts of Renilla luciferase RNA (representing HCV RNA amounts) to Firefly luciferase RNA (controlling for transfection efficiency) were quantified by real time PCR using Sso Fast™ EvaGreen Supermix (Bio-RAD, Mississauga, ON, Canada) according to manufacturer's protocol. The gene specific primers used were 5' TCGCCAGTCAAGTAACAAC 3' and 5' ACTTCGTCCACAAACACAA 3' to amplify firefly luciferase cDNA and 5' AACGCGGCCTCTTCTTATTT 3' and 5' GTCTGGTATAATACACCGCG 3' for Renilla Luciferase found in HCV replicion genome (Jopling. *et al.,* 2008). QRT-PCR was carried out using CFX 96 ™ system (Bio-RAD, Mississauga, ON, Canada).

Table 2-6. EvaGreen Gene Expression Assay reaction mix:

Component	**Volume (µl) / Reaction**
SsoFast™ EvaGreen Supermix	10 µl
Forward primer	250 nM
Reverse primer	250 nM
DNA template	Variable
Nuclease-free water	Variable
Total Volume:	20.0

Table 2-7. Optimized qRT-PCR thermal cycling conditions for EvaGreen Gene Expression Assay:

Cycling Step	Temperature	Time	# Cycles
Enzyme activation	95° C	30 s	1
Denaturation	95° C	5 s	40
Annealing/Extension	60° C	1 min	

Relative quantification was analyzed with the Bio-Rad CFX manager software (Bio-RAD, Mississauga, ON, Canada) using ΔΔCt method. The relative transcript level of each treatment was determined by a formula as follow:

$$\text{Ratio} = (E_{target})^{\Delta Ct \text{ target (Control-treated)}} / (E_{reference})^{\Delta Ct \text{ reference (Control-treated)}}$$

E represents the efficiency for the primers; ΔCt represents the difference in the Ct values, which is the point at which the fluorescence crosses the threshold. Human GAPDH was used in this study as the reference gene (Pfaffl, *et.al*. 2001).

2.11.2.3 miR-122 qRT-PCR.

For qRT-PCR analysis of endogenous miR-122 amounts following Dicer and TRBP knock down, 1 µg of total RNA was reversed transcribed *in vitro* to cDNA by miR-122 (lot: 0810096-B) and small nuclear RNA U6 (U6 snRNA) (lot: 090290-C) specific RT primers provided by TaqMan® MicroRNA Reverse Transcription Kit (*Applied Biosystems,* Carlsbad, *CA,* USA), according to manufacturer's instructions.

Table 2-8. TaqMan® MicroRNA Reverse Transcription master mix:

Component	Volume (µl) / Reaction
dNTP mix (100 mM)	0.15
Multiscribe™ RT enzyme (50 U/µL)	1.00
10 x RT Buffer	1.50
RNase Inhibitor (20 U/µL)	0.19
Nuclease free water	4.16
Gene specific primer (5 x)	3.00
Total RNA	1-10 ng
Total Volume:	15.00

The reverse transcription was incubated at 16° C for 30 min and 42° C for 30 min, and the reverse transcriptase was heat-inactivated at 85° C for 5 min. The cDNA products were stored at -20° C for further analysis.

QRT-PCR reaction was set up and performed according to protocols described in TaqMan Gene Expression Assay (9.2.1) with specific probes for miR-122 (lot: 0810096-B) and U6 snRNA (lot: 090290-C).

2.11.3 Total RNA Northern Blot:

8 µg of total RNA was denatured by using 2.7 µl deionized glyoxal (8.8 M; Sigma, Oakville, ON, Canada), 8.5 µl DMSO and 1.6 µl 0.1 M (pH 7.0) Sodium Phosphate buffer at 55º C for 1 h. Following denaturation the RNA samples were separated in 0.8% denaturing agarose gel (0.1 M NaH_2PO_4, 0.1 M $NaHPO_4$, 0.05 M EDTA, (0.1% DEPC

treated) H_2O, pH 7.0) and then transferred to Hybond N+ membrane (Amersham Pharmacia Biotech, *Québec,* Canada). The membrane was hybridized overnight in 1 x hybridization solution (5 x SSC, 5 x Denhardts solution, 1% SDS, 0.1% DEPC H_2O), 100 µg/ml denatured salmon sperm DNA (Sigma*,* Oakville, ON, Canada*)* with random-primed ^{32}P-labeled DNA probes complementary to 2940 kb fragment cut by EcoR V and BamH I from pSGR-J6/JFH-1-RLuc and to 1.3 kb fragment cut by PST1 from pUC8 hGAPDH. Following hybridization, the membrane was washed twice in wash buffer 1 (1x SSC, 1% [w/v] SDS) for 45 min and twice in wash buffer 2 (0.1 x SSC, 0.1% [w/v] SDS) for 45 min at 65°C and then exposed to a phosphorimaging screen (Bio-RAD, Mississauga, ON, Canada) overnight. Blots were quantified with Molecular Imager® (Bio-RAD, Mississauga, ON, Canada).

2.12 Statistical Analysis

All data are shown as mean ± SD of three independent experiments. Results were analyzed for statistical differences using Student's *t* test. A *p* value of ≤ 0.05 was considered to be statistically significant.

3.0 RESULTS

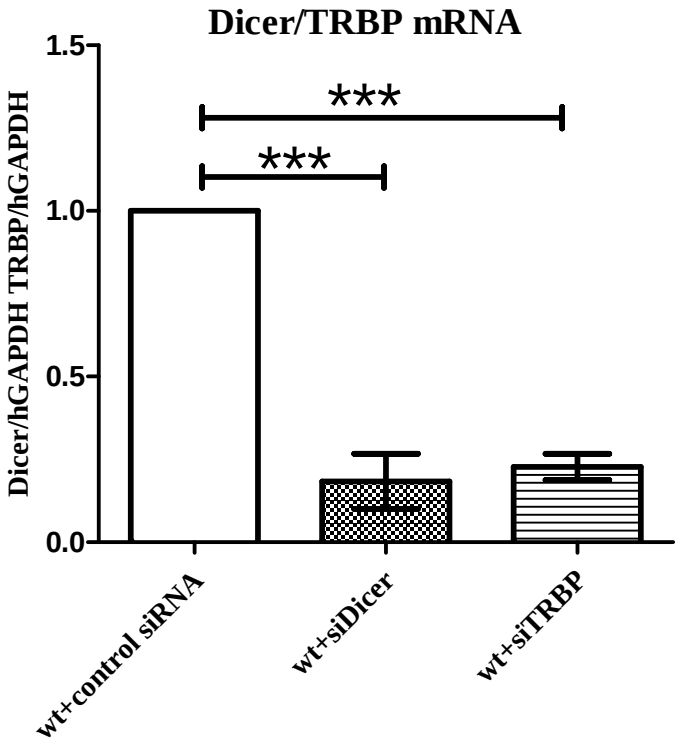
3.1 siRNA knock-down of Dicer and TRBP: mRNA, protein levels, cell viability

To further test our siRNA efficacies, total cellular RNA was extracted from Huh7.5 cell lysates at 72 h p.s.e. and quantitated by qRT-PCR for the amounts of Dicer and TRBP mRNAs. Human GAPDH serves as an internal control. The data in Figure 3-1A show that both Dicer and TRBP mRNAs in Huh7.5 cells were strongly reduced by respective siRNA treatments, compared to the control siRNA.

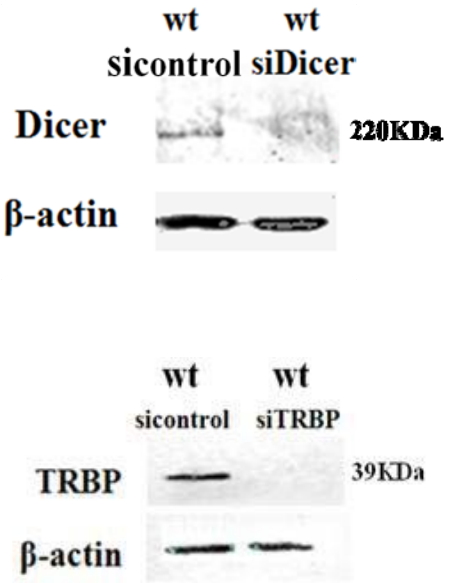
To evaluate the activities of the synthetic siRNAs in decreasing the expression of Dicer and TRBP proteins, infected Huh7.5 cell extracts were separated on SDS-PAGE and amounts of Dicer and TRBP proteins were analyzed by Western Blot at 72 h p.s.e, and normalized to human β-actin. SiDicer and siTRBP induced almost complete inhibition of protein expression, compared to control siRNA (Figure 3-1B). Since knocking down either Dicer or TRBP was assumed to destabilize the association of both proteins and a substantial reduction in the accumulation of mature miRNAs (Chendrimada, *et al.,* 2005), we suspected that knock-down of Dicer and TRBP would block the miR-122 biogenesis pathway and further interrupt HCV replication regulated by miR-122.

Since Dicer and TRBP are two essential proteins responsible for miRNA maturation in mammalian cells, their knock-down could induce a global down-regulation of miRNA and possible side effects including alterations of pivotal functions in cellular physiology, such as cell proliferation and apoptosis. To determine whether transient reductions of Dicer and TRBP induces cytotoxic effects in the context of HCV infection,

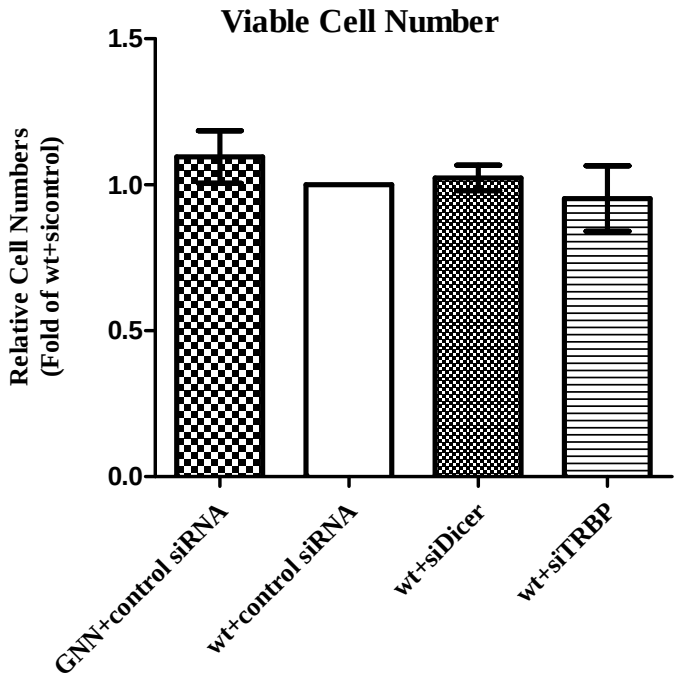
we measured the metabolic activities of viable infected cells 72 h following Dicer and TRBP knock-down by using WST-1 cell proliferation assay. WST-1 is a tetrazolium salt which can be cleaved by mitochondrial dehydrogenases, upon cleavage it releases a florescent dye whose wave length can be conveniently measured on an ELISA plate reader. The assay works on the premise that viable cell's mitochondrial dehydrogenases have equivalent activity. Therefore equivalent cell numbers will give a similar absorbance. For the purpose of our experiments a standard curve was made and the cell numbers of our samples are extrapolated off the curve. The results showed that knock-down of Dicer and TRBP had no appreciable effects on cell viability in our transient HCV replication assay, compared with cells treated with control siRNA (Figure 3-1C). Our observations agreed with Randall's work that there is no correlation between the effect of disrupting Dicer in the context of HCV infection and cell viability, suggesting that reduced HCV replication do not result from changes in cellular physiology (Randall, *et al.,* 2007). In addition, our observation was indirectly supported by the finding that down regulation of Dicer and TRBP in transient HIV infection of HeLa cells did not affect cell viability (Christensen, *et al.,* 2007).


Dicer/TRBP mRNA
Dicer/hGAPDH TRBP/hGAPDH
1.5
1.0
0.5
0.0
***
***
wt+control siRNA
wt+siDicer
wt+siTRBP

(A)


wt
wt
sicontrol
siDicer
Dicer
220KDa
β-actin
wt
wt
sicontrol
siTRBP
TRBP
39KDa
β-actin

(B)


Viable Cell Number
Relative Cell Numbers
(Fold of wt+sicontrol)
1.5
1.0
0.5
0.0
GNN+control siRNA
wt+control siRNA
wt+siDicer
wt+siTRBP

(C)

Figure 3-1: Cellular Dicer and TRBP mRNA amounts, protein expression and cell viability following siRNA knockdown.

(A): The effects of siRNAs on target gene expression were assessed by qRT-PCR at 72 h p.s.e. Human GAPDH was used as an endogenous control. Values are expressed as fold of mRNA inhibition in specific siRNA treated cells versus control siRNA treated cells.

(B): Total Huh7.5 cell lysates were harvested at 72h post p.s.e. and separated on SDS-PAGE. The protein levels of Dicer and TRBP were analyzed by Western Blot with specific antibodies. Human Dicer is about 217 kDa and was detected by anti-Dicer 14601 (Abcam) at a 1/100 dilution. Human TRBP2 is about 37 kDa and was detected with anti-TRBP42018 (Abcam) at a 1/500 dilution. Human β-actin served as an endogenous control. The blots shown above are representative results. The blots shown above were generated from one independent experiment.

(C): The numbers of viable cells were measured by WST-1 assay at 72 h p.s.e. The cell numbers of FL J6/JFH-1-RLuc-WT + control siRNA treated sample were set at 1. Values are presented as fold of the control sample.

3.2 siRNA knockdown of Dicer and TRBP attenuated Renilla Luciferase expression and HCV RNA abundance.

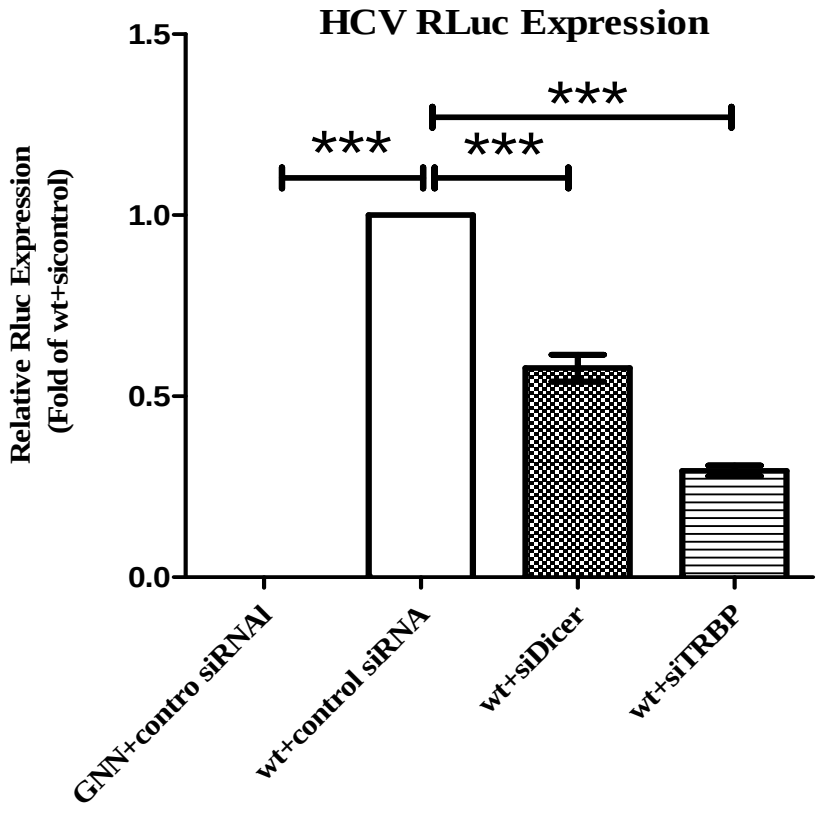
It was previously shown that, siRNA-mediated knockdown of Drosha, DGCR8, Dicer, or each of the four Argonaute proteins significantly diminished the accumulation of viral RNA in HCV-infected cells (Randall, *et al.,* 2007); however, the mechanisms explaining why and how these host factors influence HCV replication remained unknown. Since Dicer and TRBP are two essential RISC proteins responsible for miRNA biogenesis and they associate with Ago2, we considered the possibility that Dicer and

TRBP might be required for miR-122 to promote HCV infection. In this study, we investigated the specific roles of Dicer and TRBP on HCV replication.

To analyze the effects of knocking down human Dicer and TRBP on HCV replication we measured the amount of Renilla luciferase expression from the reporter virus RNA J6/JFH-1-RLuc-WT. Using this construct the levels of RLuc expression correlates with the levels of HCV RNA in the cell. For these experiments siDicer and siTRBP were introduced into Huh7.5 cells 3 days before a subsequent electroporation with J6/JFH-1-RLuc-WT HCV genome (Figure 2-1A), or J6/JFH-1-RLuc-GNN (Figure 2-1B), and the same amount of siRNA**.** Cell lysates were harvested at 1 h, or 72 h p.s.e. and RLuc activities were measured with Renilla Luciferase Assay reagent (Promega). RLuc levels were corrected for electroporation efficiency by normalizing to the RLuc expression levels at 1 h p.s.e. (before HCV replication has initiated). The RLuc expression from J6/JFH-1-RLuc-GNN RNA was a negative control in our experimental design. Knocking down Dicer reduced relative HCV RLuc expression by 42.22%, and siTRBP strongly reduced HCV RLuc expression by 70.59%, compared with control siRNA treated HCV-infected cells (Figure 3-2A).

We confirmed the reduction in HCV replication by analyzing HCV RNA abundance by using Northern Blot. Total cellular RNA was isolated from the samples in the Transient HCV Replication Assay. HCV and human GAPDH bands were probed by random-primed ^{32}P-labeled DNA fragment complementary to 2940 kb fragment cut by EcoR V and BamH I from pSGR-J6/JFH-1-RLuc and to 1.3 kb fragment restricted with PST1 from pUC8 hGAPDH, respectively. Human GAPDH was quantified to normalize signals for equal loading. Northern Blot showed that HCV RNA abundance was reduced

by 48.12% by knocking down Dicer (Figure 3-2B) and knocking down TRBP resulted in 69.55% reduction of HCV RNA accumulation (Figure 3-2C), compared to the J6/JFH-1-RLuc-WT RNA treated with control siRNA. RNA samples from J6/JFH-1-RLuc-GNN treated with control siRNA were below detectable level. Our Dicer knockdown results agree with the conclusions of Randall *et al*. (2007); however, they showed at least 75% reduction in the HCV RNA amount. Although, both of our Dicer knockdown efficiencies similarly achieved to approx 80% within Huh 7.5 cells, Randall *et al.* quantified the intracellular HCV RNAs at 48 h after siRNA electroporation and HCV infection with a multiplicity of 0.5 infectious HCV particles per cell. Therefore, we believe the different results we observed might be due to different experimental designs.



(A)

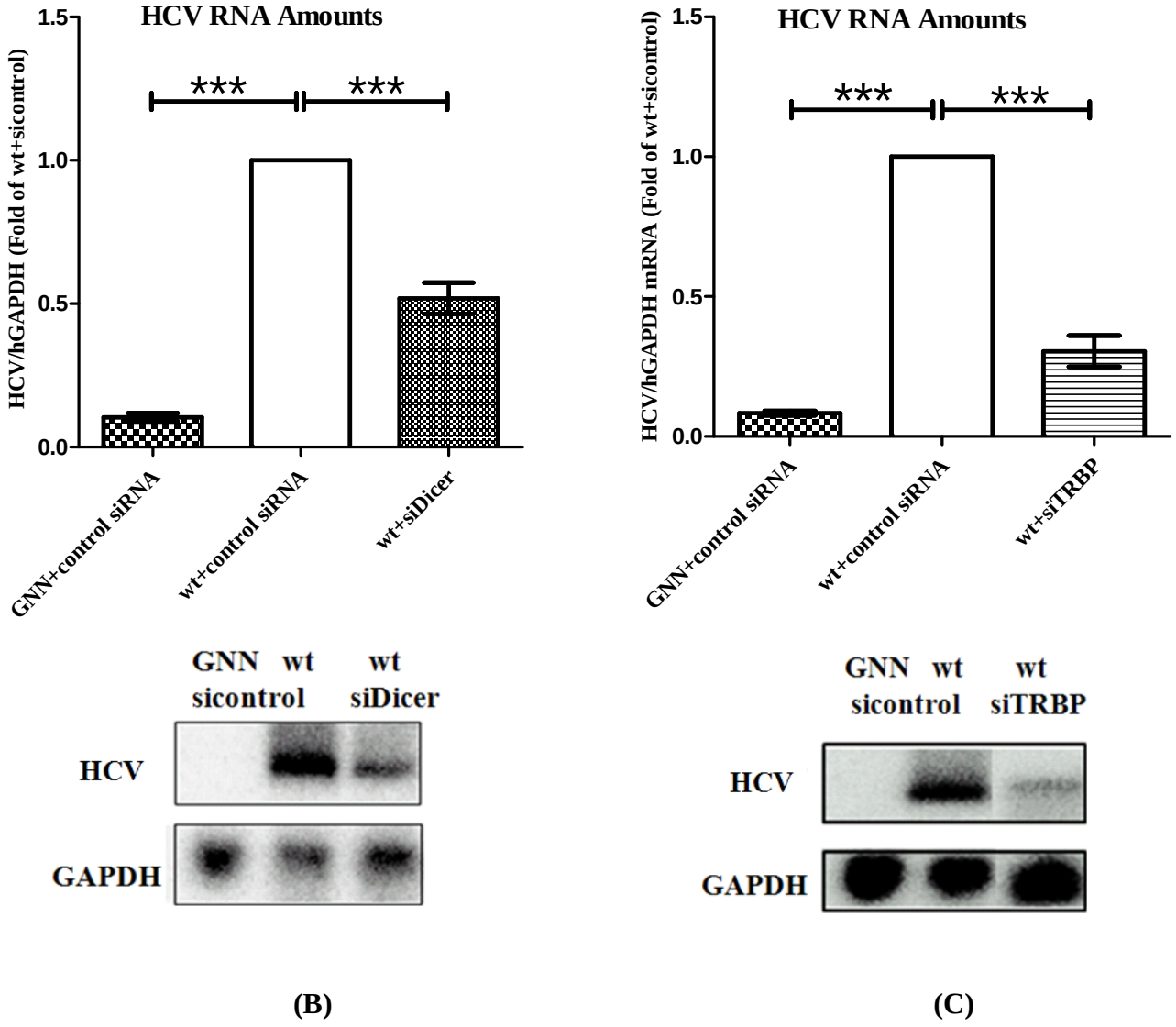


Figure 3-2: RLuc expression from HCV RLuc reporter viruses and HCV RNA amounts was reduced following siRNA knock-down of Dicer and TRBP.

(A): Relative RLuc expression of HCV replication assays at 72 h p.s.e. RLuc expression levels were reduced following Dicer and TRBP knockdown. RLuc expression levels for each sample are presented relative to control siRNA sample. Electroporation efficiency was corrected by using 1 h RLuc values. All data are shown as mean ± SD of three independent experiments. Results were

analyzed for statistical differences using Student's *t* test. A *p* value of ≤ 0.05 was considered to be statistically significant. *p≤0.05 **p≤0.01 ***p≤0.001

(B) (C): RNA abundance in HCV replication assays were quantitated by Northern Blot. HCV RNA abundance was attenuated by 48.12% by knocking down Dicer (B) and knocking down TRBP resulted in 69.55% reduction of HCV RNA accumulation (C). HCV RNA amounts were normalized to those of human GAPDH. The blots shown above are representative results. Values were graphed and expressed as fold of FL J6/JFH-1-RLuc-WT + control siRNA treated sample.

3.3 Endogenous miR-122 amounts in HCV-infected cells treated with siDicer and siTRBP

Given that Dicer and TRBP are essential proteins for miRNA biogenesis, we next inspected endogenous miR-122 amounts in our replication assays to explore whether the reduction of HCV replication could be related to diminished miR-122 abundance. We isolated total RNA from a HCV transient replication assays following Dicer and TRBP knock-down and measured levels of miR-122 at 72 h p.s.e. To quantify levels of miR-122 we used a TaqMan MicroRNA Assay Kit (Applied Biosystems) specific for miR-122. For quantification of miR-122 we reverse transcribed endogenous mature small RNAs by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and then real time quantitative PCR was carried out by using specific primers for miR-122. Small nuclear RNA U6 (U6 snRNA) served as an endogenous control, due to its stable expression and relatively constant levels across human tissues and cell lines (Peltier, *et al.,* 2008; Jopling, *et al*., 2006). Unexpectedly, endogenous miR-122 amounts were not

significantly downregulated by repressing Dicer, and levels were decreased by 25.7% following TRBP knockdown, compared with control siRNA treated sample (Figure 3-4).

We speculated that we did not see a reduction in miR-122 levels following Dicer knockdown due to the high amounts in the cell, since miR-122 accounts for approximate 70% of the total liver miRNA population and it is expressed at a high level with more than 66,000 copies per cell (Jopling, *et al*., 2005; Girard, *et al*., 2008). Sekine and co-workers reported that miR-122 could be almost depleted in hepatocyte-specific *Dicer1* conditional knockout mouse 3 weeks after birth (Sekine, *et al.,* 2009). And the half-life of mature miR-122 half life is estimated to last well beyond 24 h (Gatfield, *et al.,* 2009). Therefore, our observation could also be ascribed to the short duration of knock-down in our transient HCV replication assay that has almost no effect on high stability mature miR-122 levels. However, these data inspired us to hypothesize that the effects of Dicer and TRBP on HCV replication are not solely due to endogenous miR-122 activities and levels.

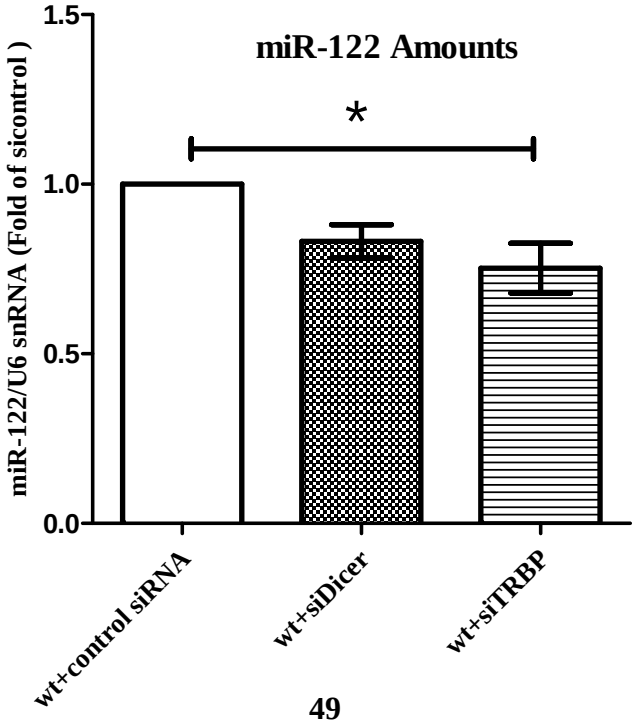


Figure 3-3: Knockdown Dicer and TRBP did not affect endogenous miR-122 levels

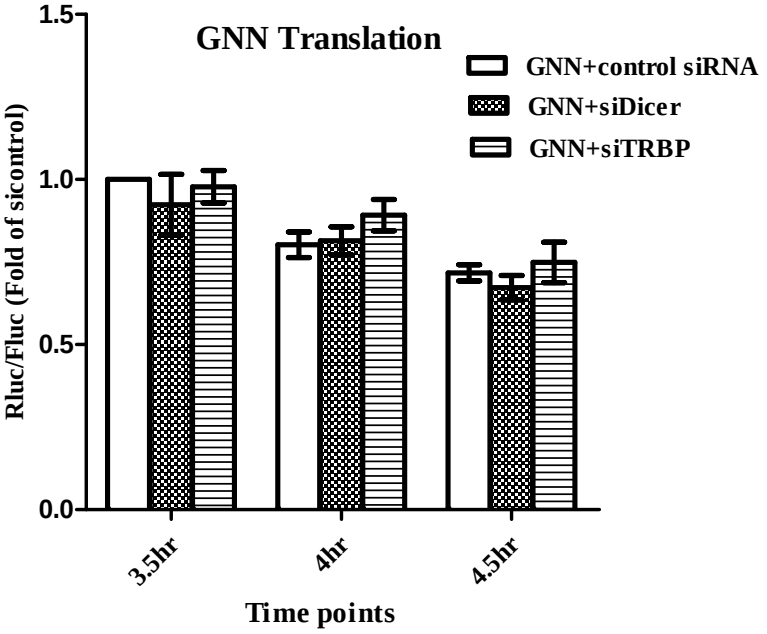
Endogenous miR-122 amounts were measured by qRT-PCR at 72 h p.s.e. Human U6 snRNA was used as an endogenous control. Values are expressed as fold of control siRNA sample.

3.4 Knocking down Dicer and TRBP had no effects on HCV translation

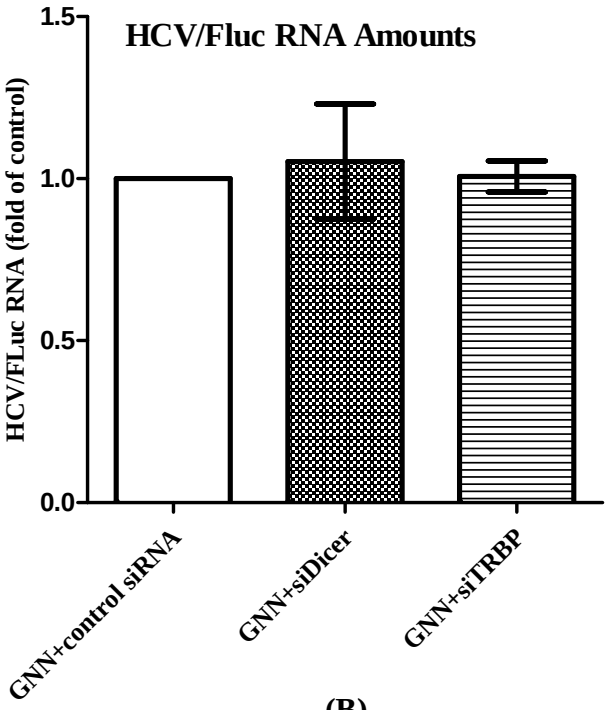
The results described above indicate that Dicer and TRBP facilitate HCV replication, but leave unresolved the mechanism by which this occurs. A previous report showed that miR-122 stimulates HCV translation through interacting with two seed-sequence binding sites in the 5'UTR (Henke, *et al.,* 2008). Thus, it is possible that the impaired HCV replication observed following Dicer and TRBP knockdown stems from a requirement of Dicer and TRBP functions in HCV translation and replication mediated by miR-122.

To explore the possibility that disrupting Dicer and TRBP expression could potentially impair HCV translation and thus viral protein synthesis at early times of HCV infection, we designed HCV translation assays to monitor the effects of knocking down Dicer and TRBP on HCV translation. Experimental approaches were similar to the transient HCV replication assay, except a replication-defective genome full length J6/JFH-1-RLuc-GNN was used as a model for studying viral translation activity. Briefly, Dicer and TRBP were pre-knocked down by 60 mM siRNAs 3 days before a second electroporation. On the second electroporation, cells were co-electroporated with the same amount of siRNAs, 1 μg a capped Firefly Luciferase (FLuc) reporter and 5 μg J6/JFH-1-RLuc-GNN HCV RNA. The FLuc reporter was used to analyze global effects in capped translation and RLuc was used to analyze the levels of HCV IRES translation, and the ratio of RLuc/FLuc indicates the relative level of HCV translation normalized to

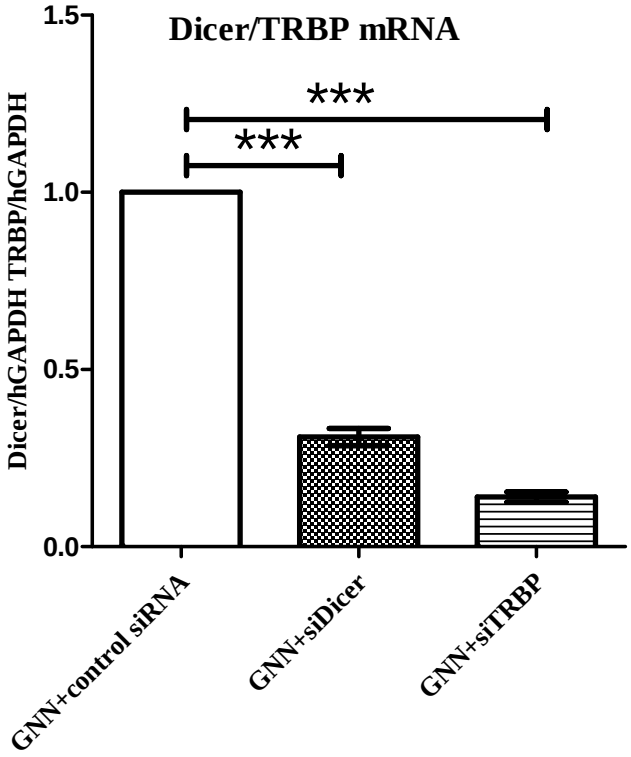
the levels of total cellular translation. Cell lysates were harvested at 3.5 h, 4 h, and 4.5 h p.s.e. Ratios of RLuc to FLuc expression indicated that decreasing Dicer and TRBP expression had no significant effects on transient HCV translation at each time point when compared with capped translation (Figure 3-4A). To exclude that relative reporter RNA levels contributed to HCV transient translation efficiency, HCV RNA and FLuc mRNA amounts were measured by qRT-PCR at 4 h p.s.e. No significant changes in the ratio of HCV/FLuc RNA were observed among the samples (Figure 3-4B). SiRNA efficacy was also tested by qRT-PCR at 4 h p.s.e. (Figure 3-4C). In addition, cell viabilities were similar in each sample as measured by WST-1 assay at 24 h p.s.e. (Figure 3-4D). Taken together, the data indicated that knocking down Dicer and TRBP had no effects on transient HCV translation. Therefore, the striking reductions in HCV replication, as measured by RLuc expression and Northern blots following Dicer and TRBP knock-down cannot be attributed to their effects on IRES directed translation in the presence of miR-122. Human Dicer and TRBP facilitate HCV infection mainly through their functions exerted on HCV replication.


GNN Translation
GNN+control siRNA
GNN+siDicer
GNN+siTRBP
Rluc/Fluc (Fold of sicontrol)
1.5
1.0
0.5
0.0
3.5hr
4hr
4.5hr
Time points

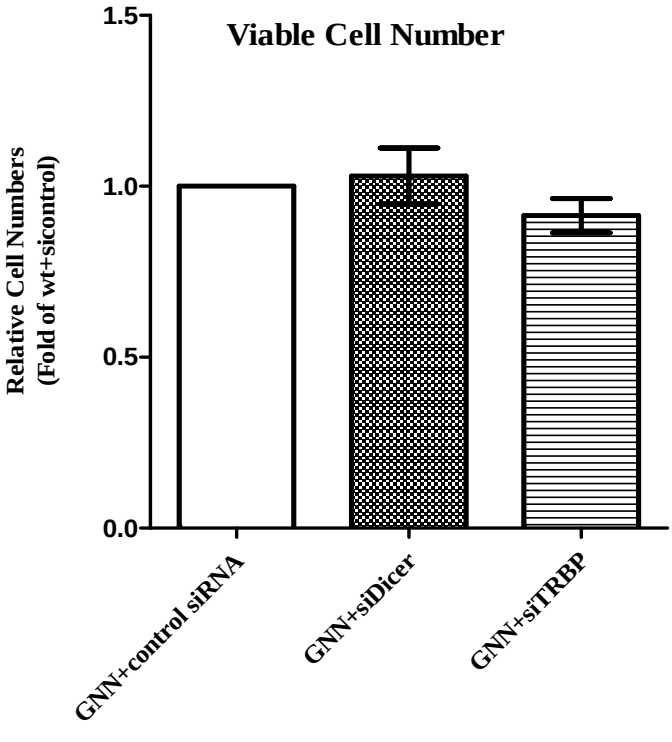
(A)


HCV/Fluc RNA Amounts
HCV/FLuc RNA (fold of control)
1.5
1.0
0.5
0.0
GNN+control siRNA
GNN+siDicer
GNN+siTRBP

(B)


Dicer/TRBP mRNA
Dicer/hGAPDH TRBP/hGAPDH
***
***
1.5
1.0
0.5
0.0
GNN+control siRNA
GNN+siDicer
GNN+siTRBP

(C)


Viable Cell Number
Relative Cell Numbers
(Fold of wt+sicontrol)
1.5
1.0
0.5
0.0
GNN+control siRNA
GNN+siDicer
GNN+siTRBP

(D)

Figure 3-4: HCV Translation assay following siRNA knock-down of Dicer and TRBP.

(A). Knocking down Dicer and TRBP did not affect HCV transient translation. Standardized RLuc/FLuc values for each sample were normalized against control siRNA. Cell lysates were harvested at 3.5 h, 4 h, and 4.5 h p.s.e.

(B). HCV RNA and FLuc reporter mRNA amounts were measured by qRT-PCR at 4 h p.s.e. with HCV and FLuc specific RT primer, as well as RLuc and FLuc specific qPCR primers (Jopling *et al.,* 2008). Standardized RLuc/FLuc values for each sample were normalized against control siRNA. The gene expression study was analyzed by CFX 96 ^{TM} system (Bio-RAD) using ΔΔCt method.

(C) Dicer and TBRP mRNA levels were measured by qRT-PCR at 4 h p.s.e. (D). Cell viabilities in parallel samples were measured by WST-1 assay at 24 h p.s.e.

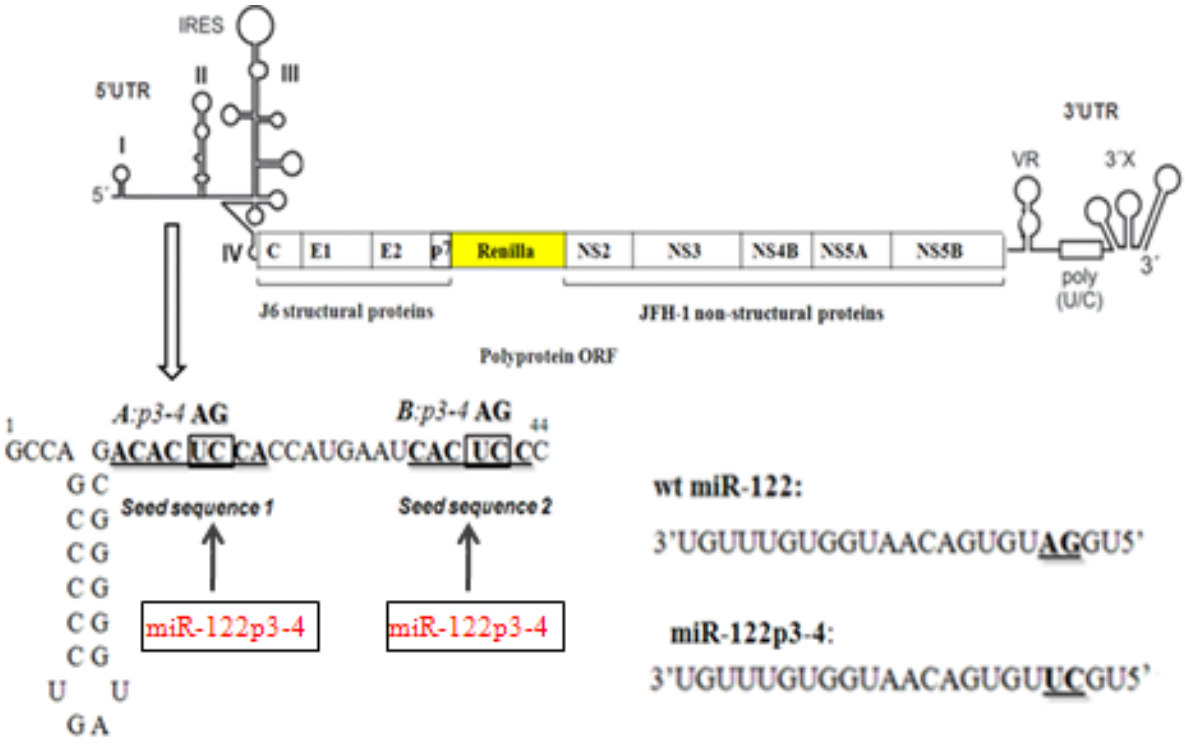
3.5 The effects of Dicer and TRBP on HCV replication are not solely due to miR-122 biogenesis, and may be due to RISC loading.

Our previous data showing that miR-122 levels did not decrease substantially in our transient HCV replication assays following Dicer and TRBP knockdown. This suggested that miR-122 levels were not responsible for reduced HCV replication. To confirm that the decrease in HCV replication is not due to reduced miR-122 levels caused by reduced miR-122 biogenesis following Dicer and TRBP knock-down, we created a mutant virus, with which replication of the virus is independent of endogenous miR-122, and dependent on a exogenously provided mutant miRNA. Using this system we can study the effects of Dicer and TRBP knockdown on HCV replication independent of any induced changes in endogenous miR-122 levels. For this assay, we used information from

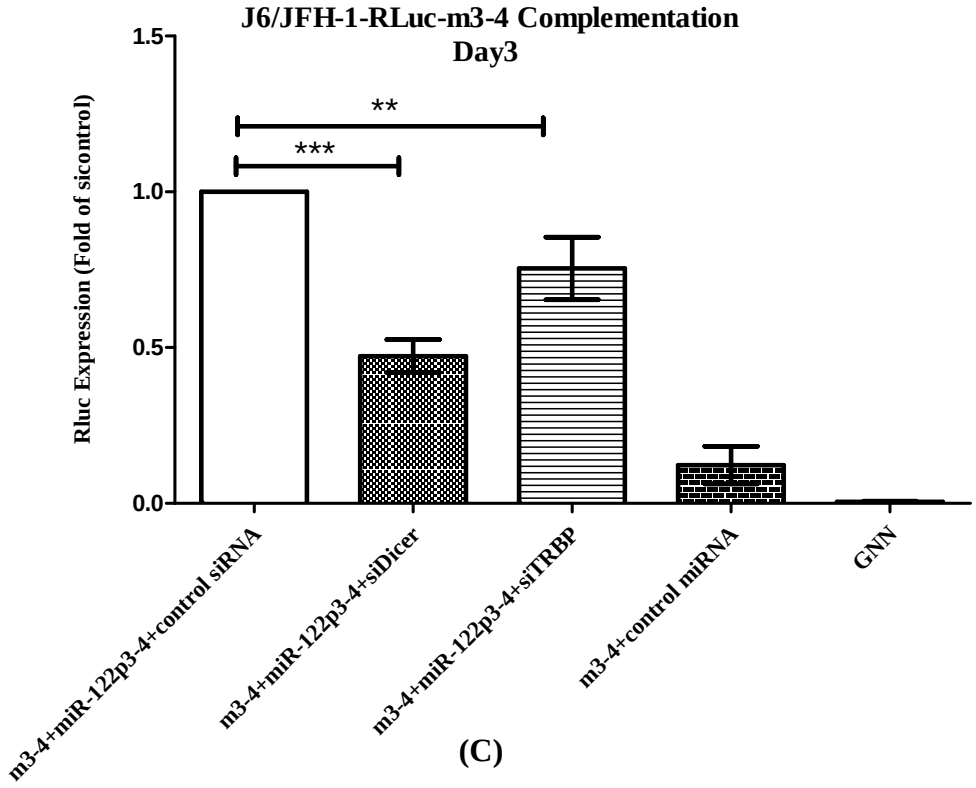
Jopling *et al.* to design a mutant HCV genome in which the miR-122 binding site seed sequences had been altered. Their group had successfully showed that exogenous mutant miR-122 (miR-122p3-4) could restore H77c (genotype 1a) RNA accumulation of genomes containing complementary mutations in the miR-122 seed binding sequences in the virus 5'UTR (Jopling, *et al*., 2006; Jopling, *et al*., 2008). Thus we mutated the nucleotides at positions 3 and 4 of each miR-122 seed sequence on the HCV genome (i.e., U26C to A27G and U41C to A42G) to yield J6/JFH-1-RLuc-m3-4 (Figure 3-5A), such that the mutant sites are not predicted to bind wt miR-122. We also synthesized a mutant miR-122p3-4 duplex, whose sequence matches the mutant binding sites on J6/JFH-1-Rluc-m3-4 such that base pairing will be restored between the miRNA and the HCV genomic RNA (Figure 3-5B). In this system, the effects of gene knockdown on HCV replication are thus independent of any possible effects on the biogenesis, and thus changes in the miR-122 levels in the cell.

Using this system, we observed that the RLuc expression from cells electroporated with J6/JFH-1-RLuc-m3-4 and control miRNA was similar to that of J6/JFH1-RLuc-GNN confirming that J6/JFH-1-RLuc-m3-4 cannot replicate without the addition of a complementary miRNA. Addition of miR-122p3-4 to cells electroporated with J6/JFH-1-RLuc-m3-4 stimulated HCV RLuc levels to about 10% of that seen with wild type virus (data not shown). Thus replication of the mutant HCV genome is dependent on binding of the mutant miR-122p3-4. The level of RLuc expression expressed by this sample was deemed 100%. Knocking down Dicer attenuated miR-122p3-4 complemented J6/JFH-1-RLuc-m3-4 RLuc expression levelsn by 52.71% on day 3 p.e., and knocking down TRBP had a moderate impact on Luciferase expression,

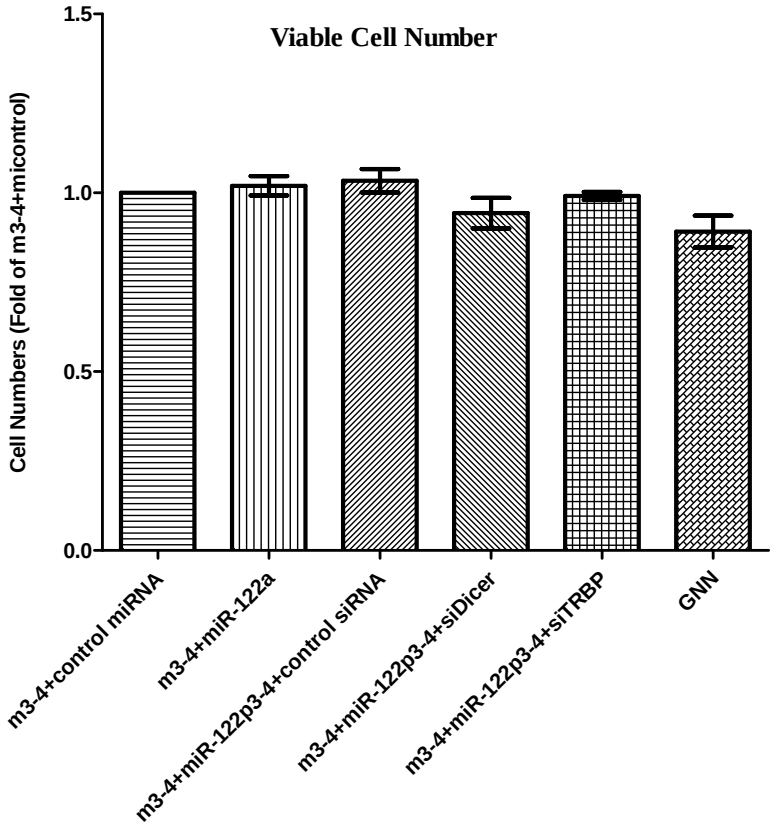
decreasing it by 24.59% on day 3 p.e. (Figure 3-5C). Cell viability was tested by WST-1 assay and did not change significantly between samples (Figure 3-5D). Therefore these results further support our hypothesis that the roles of Dicer and TRBP in HCV replication are not soley due to miR-122 biogenesis activity. Dicer and TRBP may be required for RISC assembly or for an effector step of miR-122 guided HCV augmentation. However, we cannot rule out other possible interpretations for the results described above. For example, it is possible that knocking down Dicer and TRBP could block the biogenesis of another miRNA having a role in HCV replication. Alternatively, there might exist another functional binding site for miR-122 on the HCV genome.



(A) **(B)**


J6/JFH-1-RLuc-m3-4 Complementation
Day3
Rluc Expression (Fold of sicontrol)
1.5
1.0
0.5
0.0
**
***
m3-4+miR-122p3-4+control siRNA
m3-4+miR-122p3-4+siDicer
m3-4+miR-122p3-4+siTRBP
m3-4+control miRNA
GNN

(C)


Viable Cell Number
Cell Numbers (Fold of m3-4+micontrol)
1.5
1.0
0.5
0.0
m3-4+control miRNA
m3-4+miR-122a
m3-4+miR-122p3-4+control siRNA
m3-4+miR-122p3-4+siDicer
m3-4+miR-122p3-4+siTRBP
GNN

(D)

Figure 3-5: HCV replication assay independent of endogenous miR-122 and miR-122 biogenesis.

(A): Schematic illustration of two miR-122 binding seed sequences in the 5' UTR of HCV genome, a 7-nucleotide sequence (5'ACACUCC3') and a 6-nucleotide sequence (5'CACUCC3') (underlined and presented in bold). Nucleotides at positions 3 and 4 (UC) of each seed sequence were mutated to AG to yield J6/JFH-1-RLuc-m3-4.

(B): Sequences of wt miR-122 and miR-122 p3-4 harboring complimentary mutations were commercially synthesized. Mutations were underlined and presented in bold.

(C): Knocking down Dicer and TRBP attenuates J6/JFH-1-RLuc-m3-4 replication complemented by miR-122p3-4. Values were graphed from three independent experiments and expressed as fold of J6/JFH-1-RLuc-m3-4 + miR-122m3-4 + control siRNA treated sample at each time point. Samples were harvested at 1 h, and day 3 p.e.

(D). Cell numbers in each sample were measured by WST-1 assay at day 3 p.e. Viable cell numbers were expressed as fold of J6/JFH-1-RLuc-m3-4 + control miRNA treated sample.

4.0 DISCUSSIONS

4.1 Dicer and TRBP are essential for post-transcriptional regulation of miRNA activity and contribute to HCV replication.

To elucidate the interaction between the host miRNA pathway and virus replication, previous studies have indicated that host miRNA, miR-122 and miRNA effector proteins, Argonaute 1-4, influence HCV replication (Norman, *et al.,* 2010; Randall, *et al.,* 2007). In this work, we proposed a model, in which mature miR-122 is incorporated into a ribonucleoprotein complex, similar to RISC, with the minimum constitution of miR-122 and Ago2, and miR-122 promotes HCV replication and translation through the cooperation with this complex (Figure 4-1). For this study we were interested in the roles of Dicer and TBPR, proteins that function upstream of Ago2 in the miRNA pathway, and presumably in HCV replication (Figure 4-1). In our study, we found that knockdown of human Dicer or TRBP (Figure 3-1 A, B, C), impeded HCV replication in human liver cells (Figure 3-2A, B, C) but did not affect virus translation (Figure 3-4A, B, C) or cellular proliferation (Figure 3-4D). Because Dicer and TRBP are involved in miRNA biogenesis, their knockdown is predicted to transiently block the miR-122 processing pathway. However, disrupting endogenous Dicer or TRBP did not greatly affect total amounts of miR-122 in Huh 7.5 cells (Figure 3-3). These data suggest that the half life of miR-122 is long enough that sufficient quantities are still present following 5 days of Dicer and TRBP knockdown. These data also suggest that Dicer and TRBP facilitate HCV replication in a way other than solely through their roles in miR-122 biogenesis. To further support this hypothesis, we designed an experiment in which HCV replication is dependent on an exogenously provided mutant miRNA. Thus this

assay bypasses the requirement for Dicer and TRBP in miR-122 biogenesis. For this experiment we introduced mutations to miR-122 seed-sequence binding sites in the 5'UTR of HCV (J6/JFH-1-RLuc m3-4) genome in order to prevent wt miR-122 binding (Figure 3-5A, B). The RNA abundance of mutant HCV genome (m3-4) can be restored by introducing a mutant miR-122 bearing complimentary mutations (miR-122p3-4). If Dicer or TRBP are required for activities other than miR-122 biogenesis then their knock-down will still affect HCV replication in this assay. What we found was consistent with our hypothesis: knocking down Dicer decreased miR-122p3-4 complemented J6/JFH-1 RLuc m3-4 replication 52.71% on day 3 p.e., and siTRBP attenuated this complementation process by 24.59% on day 3 p.e. (Figure 3-6 C, D). Our findings indicate that Dicer and TRBP are not only responsible for miR-122 biogenesis, but may also be involved in the activity of miR-122 in stimulating HCV replication, perhaps through the assembly of active and functional miR-122 containing RISC complex (Wang, *et al.,* 2009; Jaskiewicz, *et al.,* 2008; Gregory, *et al.,* 2005). However, we cannot exclude the possibility that the effects of Dicer or TRBP knockdown in this assay are indirect, such as via reguation of the biogenesis of a miRNA other than miR-122 that may positively influence HCV replication, or through disregulation of a host gene that contributes to HCV replication. However, these data support our hypothesis that Dicer and TRBP function to augment HCV replication through roles in both miR-122 biogenesis and through roles downstream of miR-122 biogenesis, most likely in the loading of miR-122 into the RISC complex. This also suggests that the protein complex responsible for miR-122 augmentation of HCV replication has a minimum composition of Argonaute and Dicer, and TRBP.

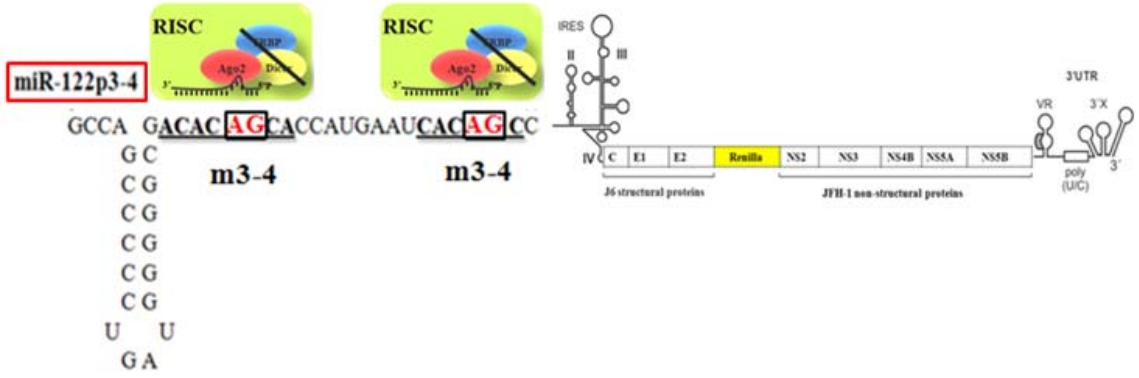


Figure 4-1: Schematic model for HCV replication assay independent of miR-122 binding and miR-122 biogenesis. The replication of J6/JFH-1-RLuc m3-4 relies on the complementation of miR-122 p3-4, as well as the possible involvement of Dicer and TRBP.

The roles of Dicer and TRBP in miRNA biogenesis have been well established (Hutvagner, *et al*., 2001; Cullen, *et al*., 2004; Chendrimada, *et al*., 2005; Jaskiewicz, *et al*., 2008). TRBP is required for efficient cleavage of pre-miRNA in vitro (Haase, *et al.*, 2005). It also assists in the miRNA processing function of Dicer through interaction with its C4 sequence within the Medipal domain in mammalian cells (Daniels, *et al*., 2009). Overexpression of TRBP1 or TRBP2 but not TRBPΔC4 in Tarbp2 $^{-/-}$cells was able to rescue processing of shRNAs or exogenous microRNAs (Daniels, *et al*., 2009). However, TRBP also has a role in assembly of the RISC. It is proposed to act as a sensor to detect proper loading of the 3'end of the guide strand onto the Ago2 PAZ domain and it also functions to orient the guide strand for direct handoff to Ago2 (Castanotto, *et al.*, 2007; Wang, *et al*., 2009). TRBP recruits Dicer complex to Ago2 for microRNA processing and gene silencing (Chendrimada, *et al*., 2005). Thus in the process of miR-122

augmentation of HCV replication, TRBP may also function to enhance miR-122 loading into Ago containing RISC.

Dicer is also thought to contribute to the process of RISC assembly. RISC is able to incorporate a Dicer-processed miRNA hairpin more effectively than a 21nt exogenous and synthetic miRNA duplex, supporting the contention that Dicer cleavage function is tightly related to the RISC loading step of miRNA gene suppression mediated by RISC (Gregory, *et al.,* 2005). It has been proposed that Ago2's Piwi domain and Mid domain interact with Dicer's platform and Ago2's N-terminal domain interacts with Dicer's base branch density during assembly of the miRNA machinery. Furthermore, the distance between the PAZ domains of Ago2 and Dicer allows a perfect accommodation of the 22nt siRNA, achieving a siRNA-bound intermediate (Wang, *et al*., 2009).

In miRNA directed translation suppression the active human RISC was shown to be composed of three proteins, Dicer, TRBP and Ago2 (Gregory, *et al.,* 2005). The Dicer–TRBP complex may be not only involved in miRNA processing but also as a platform for RISC assembly. Depletion of the Dicer–TRBP complex via exogenously introduced siRNAs resulted in a diminished siRNA-mediated Firefly Luciferase mRNA silencing (Chendrimada, *et al.,* 2005; Doi, *et al.,* 2003). The luciferase specific siRNAs did not require processing, thus the diminished silencing activity is thought to be due to inefficient RISC assembly. However, evidence suggests that Dicer might merely play a stimulatory role in the assembly and function of the mammalian RISC. One report favored that Dicer may disassociate from RISC after loading of miRNA and that Ago2 bound with miRNAs may form a functional miRNA ribonucleoproteins (miRNPs) complex by associating with other proteins without Dicer (Maniataki, *et al.,* 2005).

Moreover, following immunodepletion of Dicer, cells are still capable of retaining siRNA-mediated RISC activity (Martinez, *et al.,* 2002), and Dicer knock-out mouse embryonic stem cells are able to perform RNAi in response to exogenous siRNAs (Kanellopoulou, *et al.,* 2005).

In our studies, although endogenous miR-122 amounts were not strongly reduced by knocking down Dicer and TRBP at 72 h p.s.e., these results do not contradict the miRNA biogenesis functions of Dicer and TRBP, since other miRNA levels may have decreased, and miR-122 may persist in the cell independent of newly synthesized miRNA.

Our model for the function of TRBP and Dicer in miR-122 augmentation of HCV replication is in RISC assembly. We speculate that human TRBP may proofread the strand specificity and transfer the guide strand of miR-122 to RISC. Human Ago2 would associate with Dicer to accommodate miR-122 in a miRNA bound miRNP intermediate. Therefore, knockdown of Dicer or TRBP could result in inefficient or incorrect RISC assembly and dysfunction of miR-122 bound RISC. Previous reports have shown that knocking down either Dicer or TRBP destabilizes the associations with each other but not with Ago2 (Chendrimada, *et al.,* 2005), thus knocking down either Dicer or TRBP may abolish the activity of both proteins in the complex. Therefore Ago2 together with miRNA might be the minimum active and functional RISC in miR-122 augmentation of HCV replication.

Our data showed that Dicer and TRBP did not affect HCV translation activity. However, miR-122 has been shown to stimulate HCV translation by direct interaction with two binding sites in 5'UTR of HCV genome which enhances the association of ribosomal 48S initiation complexes and 80S ribosomes with HCV RNA (Niepmann *et al.,*

2010). Our data suggest that Dicer and TRBP may be dispensable for the miR-122 stimulation of HCV translation. But that Ago2 is involved in miR-122 stimulation of HCV translation (unpublished). Therefore, it appears that the minimum functional RISC required for miR-122 stimulation of translation is probably composed of Ago2 and miRNA.

4.2 Dicer may have competing roles when modulating HCV replication

RNAi has been shown to be a major component of the innate immune responses to viral infections in both plants and invertebrates. All RNA viruses (except retroviruses) and many DNA viruses are able to produce large amount of long, perfect dsRNA molecules in infected cells, which are recognized by cytoplasmic RNAse III Dicer as foreign. Double stranded RNA can trigger an RNAi response to generate siRNAs of viral origins, which are then used by the siRNA machinery to inhibit virus replication (Cullen, *et al.,* 2006). However, it remains unclear whether RNAi is involved in antiviral defense in mammalian cells. Since initial processing of cellular miRNA occurs in the nucleus by RNAse III Drosha, it is suggested that only viruses that replicate in the nucleus will be able to generate miRNAs. So far, evidence has failed to prove the existence of miRNAs derived from RNA viruses such as yellow fever virus and HCV in mammalian cells, (Pfeffer, *et al.,* 2005). Several DNA viruses, such as Simian Polyomavirus 40 (SV40), Herpes Simplex Virus-1 (HSV-1) and Epstein - Barr virus (EBV) (Cullen, *et al.,* 2006), were found to encode miRNA molecules in infected cells and these miRNAs are thought to help viruses establish long-term latent infections and reshape the cellular environment to maximize viral replication.

It is well established that cellular miRNAs play important roles in the regulation of cellular genes. Recent studies indicate cellular miRNAs may also have a substantial effect on the viral life cycle and may continue to influence the *in vivo* tissue tropism of viruses. Cellular miR-199 was proposed to target domain II of the IRES region in the HCV5' UTR and inhibit HCV infection (Murakami, *et al.,* 2009). In addition, 8 INF-β induced cellular miRNAs were found to antagonize HCV replication and infection, suggesting that mammals may employ cellular miRNAs to combat viral infections through the interferon system (Pedersen, *et al.,* 2007). In addition, it has been shown that the HCV core protein inhibits Dicer (Chen, *et al.,* 2008; Wang, *et al.,* 2006). However, if miRNA suppression of HCV replication was a strong effect in infected cells, then knockdown of Dicer would have caused an increase in HCV replication, instead we saw a decrease. These data suggest that miR-122 augmentation of HCV replication is dominant to any possible suppressive effect of endogenous miRNAs on HCV. However, we cannot exclude the possibility that the short duration of Dicer knock-down may not have reduced the levels of antiviral miRNA to such an extent that they affect HCV replication.

It has also been suggested that HCV RNA itself might be a substrate for Dicer. HCV IRES and its isolated domains II, III and VI were shown to be substrates and be recognized by recombinant human Dicer cleavage in EMSA experiments in vitro (Ouellet, *et al.,* 2009). HCV does not appear to elicit a RNAi response, since no miRNAs/siRNAs derived from HCV could be detected among 1318 small RNA sequences isolated from the HCV-infected Huh7.5 cell lines, and Dicer did appear to bind HCV IRES in 9-13 and Huh-7 cells, (Pfeffer, *et al.,* 2005; Ouellet, *et al.,* 2009). It has been suggested that HCV adopts a structure refractory to Dicer processing and that this resistance is modulated by

miR-122. MiR-122 was shown to induce conformational changes within the HCV IRES, when confronted with *E. coli* RNase III cleavage in *vitro*. miR-122 binding disfavors closed 'circular' conformation 'C', base paired by nt 24-38 and nt 428-442 in the core coding region (which forms long-range annealing (LRA) motif) to an open 'O' form (where bases 428–442 interact with bases 494–508 to form stem-loop VI) , which is less prone to RNase III cleavage (Beguiristain, *et al.,* 2005; Diaz-Toledano, *et al.,* 2009). Therefore, it is possible that miR-122 binds to 5' UTR of HCV genome to prevent the formation of LNA and further diverts Dicer to the core coding region, which is assumed to be less prone to Dicer processing. According to two published reports, overexpression of HCV core protein can antagonize RNA silencing and rescue HCV replication by direct interaction with Dicer (Chen, *et al.,* 2008; Wang, *et al.,* 2006); therefore, the HCV core protein could interfere with Dicer processing of HCV RNA. In addition, viral and cellular proteins interacting with the HCV IRES are likely to further limit the accessibility to Dicer processing *in vivo*. We propose that two possible ways might co-exist for human Dicer to both positively and negatively modulate HCV infection: i) promoting HCV replication through the activity of miR-122; ii) Suppressing HCV replication through the generation of anti-viral siRNAs, or host miRNAs, such as miR-199a (Murakami, *et al.,* 2009). However, the requirement of Dicer in miR-122 mediated RNAi for HCV replication must be dominant over any antiviral activity exerting against HCV since depletion of Dicer impairs HCV replication in Huh7.5 cells. It is possible that an antiviral function for Dicer might become dominant following IFN-β/γ treatments (Pedersen, *et al.,* 2007).

4.3 TRBP is an inhibitor of PKR induced antiviral pathways

In addition to its roles in miRNA biogenesis and as part of the RISC complex, TRBP also binds to and inactivates the interferon (IFN)-stimulated dsRNA-activated protein kinase (PKR) (Daher, *et al.,* 2001). The IFN-induced cellular antiviral cascade is the first defense against viral infection within an animal host. Interferon stimulation activates several antiviral pathways, one of which is the PKR pathway. Activation of PKR restricts viral replication through its ability to phosphorylate the alpha subunit of the eukaryotic initiation factor 2 (eIF-2α), resulting in a block to further cellular and viral translation (Gale, *et al.,* 1998). In order to establish a productive infection, viruses must overcome IFN-induced blocks imposed on viral replication.

PKR was shown as a key mediator of IFN action against HCV. Elevated PKR expression was reported in HCV infected hepatoma tissues and animal models (Daniels, *et al.,* 2009) and HCV replicates more efficiently in ($PKR^{-/-}$) MEF (Chang, *et al.,* 2006). It is well documented that HCV employs several strategies to counteract the antiviral effects of IFN; among them, HCV IRES, NS5A and E2 function to antagonize the translation-inhibiting function of PKR. Garaigorta *et al.* (2009) proposed that PKR activation and eIF-2α phosphorylation prevent protein synthesis of antiviral interferon-stimulated gene (ISGs) expression in the liver, but not HCV proteins. HCV-IRES was suggested to escape eIF-2α phosphorylation by recruiting ribosomes to initiate translation and bypass the hydrolysis of GTP by eIF2 (Shimoike, *et al.,* 2009). HCV NS5A and E2 proteins were shown to inhibit PKR-activation by binding "PKR-binding domain" of NS5A and PKR/eIF-2α phosphorylation homologous domain (PePHD) of E2. These may consequently release the inhibition of both cap- and HCV IRES-dependent translations

and permit infected cells to maintain persistent growth and infection that are characteristic of HCV (Gerotto, *et al.,* 2000).

In addition to its role in the process of miRNA gene suppression activity (Chendrimada, *et al.,* 2005; Daniels, *et al.,* 2009), TRBP also interacts with PKR and blocks its inhibitory effect on HIV replication (Ong, *et al.,* 2005; Daher, *et al.,* 2001). Therefore, it is possible that TRBP knockdown attenuates HCV replication through activation (or de-inhibition) of PKR. However, our data does not support this notion since knock down of TRBP did not affect HCV translation and global cap-dependent translation (Figure 3-5).

We also speculate that TRBP might bind to the HCV IRES. It is known that TRBP binds to double stranded RNA, and binds to the highly structured TAR RNA of HIV. HCV-IRES was mapped to have high-affinity PKR-IRES RNA interaction to stem-loops III-IV (Shimoike, *et al.,* 2009), which led us to hypothesize that this region might also bind TRBP. If this is the case then TRPB could protect HCV from PKR by competitive binding to the structured HCV RNA. In addition, as discussed above, the HCV IRES may have evolved to escape Dicer processing *in vivo.* We propose that HCV may also divert TRBP from Dicer to avoid RNAi antiviral function and guarantee effective viral replication. Alternatively, HCV and human liver cells would have co-evolved with favoring HCV replication by predominantly producing miR-122 over other antiviral cellular miRNAs and virus-derived miRNAs processed by Dicer and TRBP.

Therefore, we would like to follow up our studies by further investigating the mechanisms of TRBP modulation of HCV replication, and determine if it functions through the RNAi machinery or PKR, or both. To test this hypothesis, we will measure

the levels of phosphorylation of PKR in transient HCV replication assay experiments following siRNA knockdown of TRBP. Phosporylated PKR and eIF-2α protein levels could be detected by Western Blot using specific antibodies. We could also determine if the decrease to HCV replication by knockdown of TRBP is dependent on PKR. We hypothesize that knockdown of PKR would likely enhance HCV replication. If TRBP knockdown does not attenuate HCV replication in the absence of PKR, then we can conclude that the effects of TRBP on HCV are through the activity of PKR. To confirm the interaction of PKR and TRBP in HCV infected cells we could study the interaction of TRBP and PKR in HCV-infected Huh7.5 cells by co-immunoprecipiation and co-localization. The second strategy could be pursued by monitoring the effects of overexpressed TRBP upon HCV replication or translation, compared with a control plasmid. As for HIV, a small increase in the expression of TRBP has been shown to counteract the inhibitory effects of PKR on HIV-1 LTR-driven protein expression and on HIV-1 viral replication in astrocytoma cells (Gatignol, *et al.,* 1991; Ong, *et al.,* 2005). Thus we will measure HCV RNA replication and viral protein expression stimulated by TRBP in a dose dependent manner, and determines the optimum levels of TRBP expression in Huh7.5 cells.

It is also possible that TRBP binds to the HCV genomic RNA since it is a dsRBD protein. Thus we also propose to study the interaction of TRBP and HCV genome by a mobility shift assay, or RNA immunoprecipitation assay, and further map the specific domains for the protein and viral RNA interaction. If TRBP binds to the HCV genome then we speculate that TRBP could potentially compete with PKR for HCV genome binding.

Similarly, another target for a study on its role in modulating HCV replication is PKR Activating Protein (PACT). Similar to TRBP, PACT is a dsRBD protein identified to bind to Dicer and TRBP and facilitate miRNA biogenesis and RISC loading. Converse to TRPB which binds to, and inactivates PKR, PACT binds and activates PKR (Kok, *et al.,* 2007). PACT heterodimerizes with PKR/TRBP through its dsRBDs and Medipal domain and enhances PKR induced antiviral defense (Patel, *et al.,* 1998). However, under certain stress conditions, such as arsenite, peroxide and serum starvation-mediated stresses, the TRBP-PACT interaction was dissociated and the released PACT was free to further augment PKR phosphorylation (Daher *et al.,* 2009). Therefore, it also deserves investigation to unravel PACT's dual roles involved in RNAi mechanism and INF-PKR pathway and how this protein might also influence HCV replication.

To the best of our knowledge, this is the first study that links TRBP and Dicer with HCV replication and translation. Therefore, TRBP and Dicer could potentially become novel cellular targets for therapeutic interventions to block productive HCV replication in cells that are fully permissive for HCV infection.

5.0 FUTURE DIRECTIONS

Our study highlighted the potential of siRNA technology to identify multiple candidate proteins in RISC, such as Dicer, TRBP, Ago2, PACT and FXMR (an mRNA-binding protein involved in the pathogenesis of fragile X syndrome, has been shown to interact with Dicer and Ago-1 in mammalian cells (Jin, *et al.,* 2004) which modulate HCV replication and translation through miR-122 guided RNAi machinery. Our model system also preliminarily provided the groundwork for differentiating the identities of potential factors involved in the areas of miRNA biogenesis and major players functioning in the effector steps of RNAi machinery.

Future work will focus on determining if TRBP and Dicer physically associate with the HCV 5'UTR RNA sequences through miR-122 guided RISC binding. We plan to isolate RISC proteins by using biochemical methods such as immunoprecipiation to determine if TRBP and Dicer associate with HCV RNA. In addition, we will use the mobility shift assay or RNA immunoprecipitation assay to study the binding of Dicer and TRBP with HCV 5'UTR RNA. In addition, further investigation will be carried out to map the RNA sequence elements required for the protein binding by site directed mutagenesis of the 5'UTR, including mutations to the miR-122 seed binding sites. We will also analyze the effects of Dicer and TRBP knockdown on the production of infectious virus by treating infected cells with siDicer and siTRBP and analyzing the virus production by titration using a fluorescent focus forming assay. Based on these approaches, we will deepen and broaden our knowledge of specific roles of RISC proteins involving modulating HCV infection and explore novel antiviral targets.

6.0 REFERENCES

Agnello, V., G. Abel, et al. (1999). "Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor." Proc Natl Acad Sci U S A 96(22): 12766-71.

Ambros, V. (2003). "MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing." Cell 113(6): 673-6.

Andre, P., F. Komurian-Pradel, et al. (2002). "Characterization of low- and very-low-density hepatitis C virus RNA-containing particles." J Virol 76(14): 6919-28.

Andre, P., G. Perlemuter, et al. (2005). "Hepatitis C virus particles and lipoprotein metabolism." Semin Liver Dis 25(1): 93-104.

Asada, S., T. Takahashi, et al. (2008). "Downregulation of Dicer expression by serum withdrawal sensitizes human endothelial cells to apoptosis." Am J Physiol Heart Circ Physiol 295(6): H2512-21.

Bai, S., M. W. Nasser, et al. (2009). "MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib." J Biol Chem 284(46): 32015-27.

Bannwarth, S., L. Talakoub, et al. (2001). "Organization of the human tarbp2 gene reveals two promoters that are repressed in an astrocytic cell line." J Biol Chem 276(52): 48803-13.

Beguiristain, N., H. D. Robertson, et al. (2005). "RNase III cleavage demonstrates a long range RNA: RNA duplex element flanking the hepatitis C virus internal ribosome entry site." Nucleic Acids Res 33(16): 5250-61.

Benkirane, M., C. Neuveut, et al. (1997). "Oncogenic potential of TAR RNA binding protein TRBP and its regulatory interaction with RNA-dependent protein kinase PKR." Embo J 16(3): 611-24.

Bernstein, E., S. Y. Kim, et al. (2003). "Dicer is essential for mouse development." Nat Genet 35(3): 215-7.

Bode, J. G., E. D. Brenndorfer, et al. (2009). "Interplay between host cell and hepatitis C virus in regulating viral replication." Biol Chem 390(10): 1013-32.

Bohnsack, M. T., K. Czaplinski, et al. (2004). "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs." Rna 10(2): 185-91.

Boulant, S., P. Targett-Adams, et al. (2007). "Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus." J Gen Virol 88(Pt 8): 2204-13.

Bremer, J., T. O'Connor, et al. "Ablation of Dicer from murine Schwann cells increases their proliferation while blocking myelination." PLoS One 5(8): e12450.

Bu, Y., C. Lu, et al. (2009). "Knockdown of Dicer in MCF-7 human breast carcinoma cells results in G1 arrest and increased sensitivity to cisplatin." Oncol Rep 21(1): 13-7.

Bushati, N. and S. M. Cohen (2007). "microRNA functions." Annu Rev Cell Dev Biol 23: 175-205.

Castanotto, D., K. Sakurai, et al. (2007). "Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC." Nucleic Acids Res 35(15): 5154-64.

Chang, J., E. Nicolas, et al. (2004). "miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1." RNA Biol 1(2): 106-13.

Chang, K. S., Z. Cai, et al. (2006). "Replication of hepatitis C virus (HCV) RNA in mouse embryonic fibroblasts: protein kinase R (PKR)-dependent and PKR-independent mechanisms for controlling HCV RNA replication and mediating interferon activities." J Virol 80(15): 7364-74.

Chen, S. L. and T. R. Morgan (2006). "The natural history of hepatitis C virus (HCV) infection." Int J Med Sci 3(2): 47-52.

Chen, W., Z. Zhang, et al. (2008). "HCV core protein interacts with Dicer to antagonize RNA silencing." Virus Res 133(2): 250-8.

Chendrimada, T. P., R. I. Gregory, et al. (2005). "TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing." Nature 436(7051): 740-4.

Choo, Q. L., G. Kuo, et al. (1989). "Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome." Science 244(4902): 359-62.

Christensen, H. S., A. Daher, et al. (2007). "Small interfering RNAs against the TAR RNA binding protein, TRBP, a Dicer cofactor, inhibit human immunodeficiency virus type 1 long terminal repeat expression and viral production." J Virol 81(10): 5121-31.

Christensen, H. S., A. Daher, et al. (2007). "Small interfering RNAs against the TAR RNA binding protein, TRBP, a Dicer cofactor, inhibit human immunodeficiency virus type 1 long terminal repeat expression and viral production." J Virol 81(10): 5121-31.

Coulouarn, C., V. M. Factor, et al. (2009). "Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties." Oncogene 28(40): 3526-36.

Cullen, B. R. (2004). "Transcription and processing of human microRNA precursors." Mol Cell 16(6): 861-5.

Cullen, B. R. (2006). "Is RNA interference involved in intrinsic antiviral immunity in mammals?" Nat Immunol 7(6): 563-7.

Cullen, B. R. (2006). "Viruses and microRNAs." Nat Genet 38 Suppl: S25-30.

Daher, A., G. Laraki, et al. (2009). "TRBP control of PACT-induced phosphorylation of protein kinase R is reversed by stress." Mol Cell Biol 29(1): 254-65.

Daher, A., M. Longuet, et al. (2001). "Two dimerization domains in the trans-activation response RNA-binding protein (TRBP) individually reverse the protein kinase R inhibition of HIV-1 long terminal repeat expression." J Biol Chem 276(36):

33899-905.

Daniels, S. M., C. E. Melendez-Pena, et al. (2009). "Characterization of the TRBP domain required for dicer interaction and function in RNA interference." BMC Mol Biol 10: 38.

Daviet, L., M. Erard, et al. (2000). "Analysis of a binding difference between the two dsRNA-binding domains in TRBP reveals the modular function of a KR-helix motif." Eur J Biochem 267(8): 2419-31.

Denli, A. M., B. B. Tops, et al. (2004). "Processing of primary microRNAs by the Microprocessor complex." Nature 432(7014): 231-5.

Diaz-Toledano, R., A. Ariza-Mateos, et al. (2009). "In vitro characterization of a miR-122-sensitive double-helical switch element in the 5' region of hepatitis C virus RNA." Nucleic Acids Res 37(16): 5498-510.

Doi, N., S. Zenno, et al. (2003). "Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors." Curr Biol 13(1): 41-6.

Dorin, D., M. C. Bonnet, et al. (2003). "The TAR RNA-binding protein, TRBP, stimulates the expression of TAR-containing RNAs in vitro and in vivo independently of its ability to inhibit the dsRNA-dependent kinase PKR." J Biol Chem 278(7): 4440-8.

Egger, D., B. Wolk, et al. (2002). "Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex." J Virol 76(12): 5974-84.

Elmen, J., M. Lindow, et al. (2008). "LNA-mediated microRNA silencing in non-human primates." Nature 452(7189): 896-9.

Elmen, J., M. Lindow, et al. (2008). "Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver." Nucleic Acids Res 36(4): 1153-62.

Esau, C., S. Davis, et al. (2006). "miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting." Cell Metab 3(2): 87-98.

Evans, M. J., T. von Hahn, et al. (2007). "Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry." Nature 446(7137): 801-5.

Farci, P. (2002). "Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome [Science 1989;244:359-362]." J Hepatol 36(5): 582-5.

Filipowicz, W., S. N. Bhattacharyya, et al. (2008). "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" Nat Rev Genet 9(2): 102-14.

Fraser, C. S. and J. A. Doudna (2007). "Structural and mechanistic insights into hepatitis C viral translation initiation." Nat Rev Microbiol 5(1): 29-38.

Fu, H., Y. Tie, et al. (2005). "Identification of human fetal liver miRNAs by a novel method." FEBS Lett 579(17): 3849-54.

Fukagawa, T., M. Nogami, et al. (2004). "Dicer is essential for formation of the heterochromatin structure in vertebrate cells." Nat Cell Biol 6(8): 784-91.

Gale, M., Jr. and M. G. Katze (1998). "Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase." Pharmacol Ther 78(1): 29-46.

Garaigorta, U. and F. V. Chisari (2009). "Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation." Cell Host Microbe 6(6): 513-22.

Garcia, M. A., E. F. Meurs, et al. (2007). "The dsRNA protein kinase PKR: virus and cell control." Biochimie 89(6-7): 799-811.

Gastaminza, P., G. Cheng, et al. (2008). "Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion." J Virol 82(5): 2120-9.

Gatfield, D., G. Le Martelot, et al. (2009). "Integration of microRNA miR-122 in hepatic circadian gene expression." Genes Dev 23(11): 1313-26.

Gatignol, A., A. Buckler-White, et al. (1991). "Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR." Science 251(5001): 1597-600.
Gatignol, A., A. Buckler-White, et al. (1991). "Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR." Science 251(5001): 1597-600.
Gatignol, A., S. Laine, et al. (2005). "Dual role of TRBP in HIV replication and RNA interference: viral diversion of a cellular pathway or evasion from antiviral immunity?" Retrovirology 2: 65.

Gerotto, M., F. Dal Pero, et al. (2000).Two PKR inhibitor HCV proteins correlate with early but not sustained response to interferon.Gastroenterology 119(6)1649-55.

Gibbons, G. F., D. Wiggins, et al. (2004). "Synthesis and function of hepatic very-low-density lipoprotein." Biochem Soc Trans 32(Pt 1): 59-64.

Giraldez, A. J., Y. Mishima, et al. (2006). "Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs." Science 312(5770): 75-9.

Girard, M., E. Jacquemin, et al. (2008). "miR-122, a paradigm for the role of microRNAs in the liver." J Hepatol 48(4): 648-56.

Gosert, R., D. Egger, et al. (2003). "Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons." J Virol 77(9): 5487-92.

Gregory, R. I., T. P. Chendrimada, et al. (2005). "Human RISC couples microRNA biogenesis and posttranscriptional gene silencing." Cell 123(4): 631-40.

Gregory, R. I., K. P. Yan, et al. (2004). "The Microprocessor complex mediates the genesis of microRNAs." Nature 432(7014): 235-40.

Haase, A. D., L. Jaskiewicz, et al. (2005). "TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing." EMBO Rep 6(10): 961-7.

Han, J., Y. Lee, et al. (2004). "The Drosha-DGCR8 complex in primary microRNA processing." Genes Dev 18(24): 3016-27.

Han, L., A. Zhang, et al. "Downregulation of Dicer enhances tumor cell proliferation and invasion." Int J Oncol 37(2): 299-305.

Harfe, B. D. (2005). "MicroRNAs in vertebrate development." Curr Opin Genet Dev 15(4): 410-5.

Harris, K. S., Z. Zhang, et al. (2006). "Dicer function is essential for lung epithelium morphogenesis." Proc Natl Acad Sci U S A 103(7): 2208-13.

Haussecker, D. and M. A. Kay "miR-122 continues to blaze the trail for microRNA therapeutics." Mol Ther 18(2): 240-2.

Hellen, C. U. (2009). "IRES-induced conformational changes in the ribosome and the mechanism of translation initiation by internal ribosomal entry." Biochim Biophys Acta 1789(9-10): 558-70.

Henke, J. I., D. Goergen, et al. (2008). "microRNA-122 stimulates translation of hepatitis C virus RNA." Embo J 27(24): 3300-10.

Heo, I. and V. N. Kim (2009). "Regulating the regulators: posttranslational modifications of RNA silencing factors." Cell 139(1): 28-31.

Huang, H., F. Sun, et al. (2007). "Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins." Proc Natl Acad Sci U S A 104(14): 5848-53.

Hutvagner, G., J. McLachlan, et al. (2001). "A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA." Science 293(5531): 834-8.

Hwang, H. W. and J. T. Mendell (2006). "MicroRNAs in cell proliferation, cell death, and tumorigenesis." Br J Cancer 94(6): 776-80.

http://www.hc-sc.gc.ca/hl-vs/iyh-vsv/diseases-maladies/hepc-eng.php.

http://www.tibotec.com/content/backgrounders/www.tibotec.com/hcv_lifecycle.html.

Isken, O., M. Baroth, et al. (2007). "Nuclear factors are involved in hepatitis C virus RNA replication." Rna 13(10): 1675-92.

Jangra, R. K., M. Yi, et al. "miR-122 Regulation of Hepatitis C Virus Translation and Infectious Virus Production." J Virol.

Jaskiewicz, L. and W. Filipowicz (2008). "Role of Dicer in posttranscriptional RNA silencing." Curr Top Microbiol Immunol 320: 77-97.

Jin, P., D. C. Zarnescu, et al. (2004). "Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway." Nat Neurosci 7(2): 113-7.

Jones, C. T., C. L. Murray, et al. (2007). "Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus." J Virol 81(16): 8374-83.

Jones, D. M. and J. McLauchlan "Hepatitis C virus: assembly and release of virus particles." J Biol Chem.

Jones, D. M., A. H. Patel, et al. (2009). "The hepatitis C virus NS4B protein can trans-complement viral RNA replication and modulates production of infectious virus." J Virol 83(5): 2163-77.

Jopling, C. L., K. L. Norman, et al. (2006). "Positive and negative modulation of viral and cellular mRNAs by liver-specific microRNA miR-122." Cold Spring Harb Symp Quant Biol 71: 369-76.

Jopling, C. L., S. Schutz, et al. (2008). "Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome." Cell Host Microbe 4(1): 77-85.

Jopling, C. L., M. Yi, et al. (2005). "Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA." Science 309(5740): 1577-81.

Kanellopoulou, C., S. A. Muljo, et al. (2005). "Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing." Genes Dev 19(4): 489-501.

Kapadia, S. B., H. Barth, et al. (2007). "Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger

receptor B type I." J Virol 81(1): 374-83.

Kato, T., T. Date, et al. (2003). "Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon." Gastroenterology 125(6): 1808-17.

Kok, K. H., M. H. Ng, et al. (2007). "Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA." J Biol Chem 282(24): 17649-57.

Krutzfeldt, J., N. Rajewsky, et al. (2005). "Silencing of microRNAs in vivo with 'antagomirs'." Nature 438(7068): 685-9.

Lanford, R. E., E. S. Hildebrandt-Eriksen, et al. "Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection." Science 327(5962): 198-201.

Laraki, G., G. Clerzius, et al. (2008). "Interactions between the double-stranded RNA-binding proteins TRBP and PACT define the Medipal domain that mediates protein-protein interactions." RNA Biol 5(2): 92-103.

Lauer, G. M. and B. D. Walker (2001). "Hepatitis C virus infection." N Engl J Med 345(1): 41-52.

Lee, J. Y., H. Kim, et al. (2004). "Merlin, a tumor suppressor, interacts with transactivation-responsive RNA-binding protein and inhibits its oncogenic activity." J Biol Chem 279(29): 30265-73.

Lee, Y., I. Hur, et al. (2006). "The role of PACT in the RNA silencing pathway." Embo J 25(3): 522-32.

Lindenbach, B. D., P. Meuleman, et al. (2006). "Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro." Proc Natl Acad Sci U S A 103(10): 3805-9.

Lindsay, M. A. (2008). "microRNAs and the immune response." Trends Immunol 29(7): 343-51.

Lohmann, V., F. Korner, et al. (1999). "Replication of subgenomic hepatitis C virus

RNAs in a hepatoma cell line." Science 285(5424): 110-3.

Lozach, P. Y., H. Lortat-Jacob, et al. (2003). "DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2." J Biol Chem 278(22): 20358-66.

Luense, L. J., M. Z. Carletti, et al. (2009). Role of Dicer in female fertility. Trends Endocrinol Metab. 20: 265-72.

Lukavsky, P. J. (2009). "Structure and function of HCV IRES domains." Virus Res 139(2): 166-71.

Ma, Y., J. Yates, et al. (2008). "NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly." J Virol 82(15): 7624-39.

MacRae, I. J., E. Ma, et al. (2008). "In vitro reconstitution of the human RISC-loading complex." Proc Natl Acad Sci U S A 105(2): 512-7.

Maniataki, E. and Z. Mourelatos (2005). A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. 19: 2979-90.

Martinez, J., A. Patkaniowska, et al. (2002). "Single-stranded antisense siRNAs guide target RNA cleavage in RNAi." Cell 110(5): 563-74.

Masaki, T., R. Suzuki, et al. (2008). "Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles." J Virol 82(16): 7964-76.

Matskevich, A. A. and K. Moelling (2007). "Dicer is involved in protection against influenza A virus infection." J Gen Virol 88(Pt 10): 2627-35.

McOmish, F., P. L. Yap, et al. (1994). "Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey." J Clin Microbiol 32(4): 884-92.

Melo, S. A., S. Ropero, et al. (2009). "A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function." Nat Genet 41(3): 365-70.

Meyuhas, O. (2000). "Synthesis of the translational apparatus is regulated at the

translational level." Eur J Biochem 267(21): 6321-30.

Miyanari, Y., K. Atsuzawa, et al. (2007). "The lipid droplet is an important organelle for hepatitis C virus production." Nat Cell Biol 9(9): 1089-97.

Moradpour, D., F. Penin, et al. (2007). "Replication of hepatitis C virus." Nat Rev Microbiol 5(6): 453-63.

Murakami, Y., H. H. Aly, et al. (2009). "Regulation of the hepatitis C virus genome replication by miR-199a." J Hepatol 50(3): 453-60.

Murchison, E. P. and G. J. Hannon (2004). "miRNAs on the move: miRNA biogenesis and the RNAi machinery." Curr Opin Cell Biol 16(3): 223-9.

Murchison, E. P., J. F. Partridge, et al. (2005). "Characterization of Dicer-deficient murine embryonic stem cells." Proc Natl Acad Sci U S A 102(34): 12135-40.

Murphy, D., B. Dancis, et al. (2008). "The evolution of core proteins involved in microRNA biogenesis." BMC Evol Biol 8: 92.

Norman, K. L. and P. Sarnow "Hepatitis C virus' Achilles' heel--dependence on liver-specific microRNA miR-122." Cell Res 20(3): 247-9.

Ong, C. L., J. C. Thorpe, et al. (2005). "Low TRBP levels support an innate human immunodeficiency virus type 1 resistance in astrocytes by enhancing the PKR antiviral response." J Virol 79(20): 12763-72.

Ouellet, D. L., I. Plante, et al. (2009). "Refractoriness of hepatitis C virus internal ribosome entry site to processing by Dicer in vivo." J Negat Results Biomed 88.

Park, H., M. V. Davies, et al. (1994). "TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR." Proc Natl Acad Sci U S A 91(11): 4713-7.

Parker, G. S., T. S. Maity, et al. (2008). "dsRNA binding properties of RDE-4 and TRBP reflect their distinct roles in RNAi." J Mol Biol 384(4): 967-79.

Paroo, Z., X. Ye, et al. (2009). "Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling." Cell 139(1): 112-22.

Patel, R. C. and G. C. Sen (1998). "PACT, a protein activator of the interferon-induced protein kinase, PKR." Embo J 17(15): 4379-90.

Pawlotsky, J. M., S. Chevaliez, et al. (2007). "The hepatitis C virus life cycle as a target for new antiviral therapies." Gastroenterology 132(5): 1979-98.

Pedersen, I. M., G. Cheng, et al. (2007). "Interferon modulation of cellular microRNAs as an antiviral mechanism." Nature 449(7164): 919-22.

Peltier, H. J. and G. J. Latham (2008). "Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues." Rna 14(5): 844-52.

Pereira, J. A., R. Baumann, et al. "Dicer in Schwann cells is required for myelination and axonal integrity." J Neurosci 30(19): 6763-75.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res 29(9): e45.

Pfeffer, S., A. Sewer, et al. (2005). "Identification of microRNAs of the herpesvirus family." Nat Methods 2(4): 269-76.

Pietschmann, T., A. Kaul, et al. (2006). "Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras." Proc Natl Acad Sci U S A 103(19): 7408-13.

Pileri, P., Y. Uematsu, et al. (1998). "Binding of hepatitis C virus to CD81." Science 282(5390): 938-41.

Ploss, A., M. J. Evans, et al. (2009). "Human occludin is a hepatitis C virus entry factor required for infection of mouse cells." Nature 457(7231): 882-6.

Provost, P., D. Dishart, et al. (2002). "Ribonuclease activity and RNA binding of recombinant human Dicer." Embo J 21(21): 5864-74.

Qureshi, S. A. (2007). "Hepatitis C virus--biology, host evasion strategies, and promising new therapies on the horizon." Med Res Rev 27(3): 353-73.

Randall, G., M. Panis, et al. (2007). "Cellular cofactors affecting hepatitis C virus

infection and replication." Proc Natl Acad Sci U S A 104(31): 12884-9.

Rivas, F. V., N. H. Tolia, et al. (2005). "Purified Argonaute2 and an siRNA form recombinant human RISC." Nat Struct Mol Biol 12(4): 340-9.

Rossi, J. J. (2005). "Mammalian Dicer finds a partner." EMBO Rep 6(10): 927-9.

Saunier, B., M. Triyatni, et al. (2003). "Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes." J Virol 77(1): 546-59.

Scarselli, E., H. Ansuini, et al. (2002). "The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus." Embo J 21(19): 5017-25.

Schwarz, D. S., G. Hutvagner, et al. (2003). "Asymmetry in the assembly of the RNAi enzyme complex." Cell 115(2): 199-208.

Schwer, B. (2001). "A new twist on RNA helicases: DExH/D box proteins as RNPases." Nat Struct Biol 8(2): 113-6.

Sekine, S., R. Ogawa, et al. (2009). "Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis." Gastroenterology 136(7): 2304-2315 e1-4.

Serafino, A., M. B. Valli, et al. (2003). "Suggested role of the Golgi apparatus and endoplasmic reticulum for crucial sites of hepatitis C virus replication in human lymphoblastoid cells infected in vitro." J Med Virol 70(1): 31-41.

Shimoike, T., S. A. McKenna, et al. (2009). "Translational insensitivity to potent activation of PKR by HCV IRES RNA." Antiviral Res 83(3): 228-37.

Spahn, C. M., J. S. Kieft, et al. (2001). "Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit." Science 291(5510): 1959-62.

Steinmann, E., F. Penin, et al. (2007). "Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions." PLoS Pathog 3(7): e103.

Suzuki, T., K. Ishii, et al. (2007). "Hepatitis C viral life cycle." Adv Drug Deliv Rev 59(12): 1200-12.

Tanaka, T., N. Kato, et al. (1996). "Structure of the 3' terminus of the hepatitis C virus genome." J Virol 70(5): 3307-12.

Tanaka, Y., T. Shimoike, et al. (2000). "Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5' untranslated region of the viral genome." Virology 270(1): 229-36.

Tsukiyama-Kohara, K., N. Iizuka, et al. (1992). "Internal ribosome entry site within hepatitis C virus RNA." J Virol 66(3): 1476-83.

Wang, B., T. M. Love, et al. (2006). "Recapitulation of short RNA-directed translational gene silencing in vitro." Mol Cell 22(4): 553-60.

Wang, H. W., C. Noland, et al. (2009). "Structural insights into RNA processing by the human RISC-loading complex." Nat Struct Mol Biol 16(11): 1148-53.

Wang, Y., N. Kato, et al. (2006). "Hepatitis C virus core protein is a potent inhibitor of RNA silencing-based antiviral response." Gastroenterology 130(3): 883-92.

White, S. A. and R. C. Allshire (2004). "Loss of Dicer fowls up centromeres." Nat Cell Biol 6(8): 696-7.

Wilson, J. A. and C. D. Richardson (2003). "Induction of RNA interference using short interfering RNA expression vectors in cell culture and animal systems." Curr Opin Mol Ther 5(4): 389-96.

Wilson, J. A. and C. D. Richardson (2005). "Hepatitis C virus replicons escape RNA interference induced by a short interfering RNA directed against the NS5b coding region." J Virol 79(11): 7050-8.

Zhang, H., F. A. Kolb, et al. (2002). "Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP." Embo J 21(21): 5875-85.

Zhang, H., F. A. Kolb, et al. (2004). "Single processing center models for human Dicer and bacterial RNase III." Cell 118(1): 57-68.

Zhong, J., P. Gastaminza, et al. (2005). "Robust hepatitis C virus infection in vitro." Proc Natl Acad Sci U S A 102(26): 9294-9.

Zhong, J., A. H. Peters, et al. (1999). "A double-stranded RNA binding protein required for activation of repressed messages in mammalian germ cells." Nat Genet 22(2): 171-4.